

Europe's parliament loses reputation

The decision of the Strasbourg parliament to throw out a draft directive on genetic manipulation will do less harm to biotechnology than to the parliament itself.

EVER since the Maastricht Treaty was eventually ratified at the end of 1993, the European Parliament has been walking taller, flexing its muscles and looking forward to the increased influence it is promised by the treaty. There are good reasons to welcome that development. The smaller members of the European Union (EU) naturally see the parliament as a valuable counterpoise to the power of the Council of Ministers (where big countries have more votes) and the European Commission, but even the German government has been a consistent advocate of more influence for the parliament, apparently on the self-denying grounds that a democracy needs an effective parliament. So, until last week, the wind seemed to be at Strasbourg's back. And then the European Parliament, in a rush of blood to the head, threw away its reputation for good sense and probity.

It is an extraordinary story (see page 103). The Commission has been struggling with the Council of Ministers for two years to devise a directive on the patentability of innovations in human, animal and plant genetics. As things stand, there is a European Patent Office (EPO) established by treaty and national legislation that reflects EPO's charter, even down to the provision that inventions should not be awarded if they are contrary to the *ordre publique*, a French concept loosely translated as unseemly and immoral.

The past few years have thrown up a host of conundrums with which patent examiners must contend, often for the first time. Are 'expressed sequence tags' patentable? Does the legitimate protection afforded to the discovery of a significant mutation in a single gene (which might become the basis of a diagnostic test) give the patent-holder the right to exploit all other mutations of the same gene? What kinds of rights does a genetic manipulator acquire over the progeny of genetically engineered plants and animals that may be sold to farmers?

The Commission's draft directive sought to clarify many of these issues, mostly uncontentionously. Partial DNA sequences should not be patentable unless they have direct utility, for example. But in the patenting of genetically modified animals (plants are dealt with differently), the test should be whether "a substantial benefit to man or animal" should offset "suffering or physical handicap" to the animals. In short, the directive is not a charter for those who would engage in genetic manipulation designed to give the European public an attack of horrors, but rather is a mildly restrictive clarification of present practice. But against rea-

son, and the odds, the parliament threw out the directive as a whole. It would have none of it.

This sombre incident says more about the European Parliament than about the present or future practice of genetic manipulation in Europe. Since its inception, the parliament has been notoriously susceptible to the influence of pressure groups, at the outset because its members needed the help that pressure groups could provide, latterly because they have become familiar. The parliament is also given to gusts of emotional reaction, which hitherto have mattered little because its role was advisory only. But now the Commission will have to pay attention to the parliament's vote. Certainly, important questions will be delayed. No doubt there will be a further round of negotiations and further compromise.

On reflection, wiser heads in Strasbourg (where the parliament spends some of its time) will conclude that this latest show of political correctness has been an act of irresponsibility, as if it were a parliament demanding social programmes from its government while refusing to sanction the taxes with which to pay for them. The point is all the more telling because the parliament has an ambition (and a right under Maastricht) to have an influence on the development of European foreign and security policy, both of them fields of public policy in which an element of realism is inseparable from success. On last week's form, it can be only a matter of time before Strasbourg is voting for the abolition of armed forces on the grounds that their members are equipped with munitions, which are dangerous. □

Unbalanced US budgets

There should be relief that the new US Congress has failed to pass its talismanic constitutional amendment.

THE United States is lucky that the famous Balanced Budget Amendment, a central part of the Republican Party's *Contract with America*, failed to pass the Senate last week. In reality, the whole project is a recipe for disaster. Last Friday, even *The Wall Street Journal* acknowledged that the project has been largely a symbolic issue, a promise to voters that politicians would never again be spendthrift with their money. But it is worse than that. Either the promise would have been hollow in the sense that politicians would have found ways around it, or it would have led to spending



cuts of the kind that those supporting the amendment would have resented deeply. And it would have made orderly government in the United States less attainable.

What follows is not political speculation (of which there is ample in the US press, notably on the theme of how the chances of the next round of presidential candidates will have been affected by the Senate's vote), but a reflection on public budgets generally. It starts from the view that the US federal budget (now, in round numbers, \$200 billion a year) is indeed too large, certainly in the present modest economic recovery, and that there are gloomy signs that the deficit, institutionalized by President Ronald Reagan, has become chronic. Why else was it so resistant to reduction during the seemingly prosperous late-1980s? The weakness of the US dollar in the past few weeks is partly a consequence of that, now that Japanese institutions and persons are no longer keeping their savings in the United States.

But the US budget a constitutional amendment would have balanced by law is, despite its size, only the tip of an iceberg. A large part of the US government's financial activity is not covered by the budget proper, but by semi-autonomous agencies spending funds borrowed from the financial markets and operated in such a way that only deficits appear as costs in the budget that must be financed by federal taxes. From mortgages for affordable housing to many of the loan schemes by which students survive through college courses (not to mention the rescue of the Savings and Loan banks a decade ago), off-budget financing is well tried and widely practised.

The promise of the Balanced Budget Amendment is potentially hollow because the limits of off-budget financing have not been thoroughly explored. Take, for example, federal spending on research, which is discretionary in the sense that no law compels the US government to spend money in this way, but which is a public good that the government needs to support and from which innovation and improved health can reliably be expected to flow. So why not transfer all these activities to a new public corporation (shades of the Galvin report on the US national laboratories, see *Nature* **373**, 463–464; 1995) that would finance them with borrowed funds and would eventually repay the money either from the sale of patents or by means of a levy on the ultimate beneficiaries? It is, of course, a bad idea, but one that future governments caught by a Balanced Budget Amendment might well embrace.

That underlines a more serious difficulty — the distinction between public investment and public spending generally in all public budgets (not only that of the United States). There are many circumstances in which a new road or harbour, or the support of students in higher education, can be reckoned as an ultimately productive tangible investment. Even allowing for the frailty of governments in deciding which investments are worthwhile, there is a case for treating such expenditure differently from what are called, in the United States, 'entitlements'. Technically, the former deserve protection; politically, the shoe is on the other foot. And that is the crux of the budget problem in the United States: the federal budget will not be balanced painlessly,

but only by transferring the burden either to state governments or to taxpayers in general. The project for a constitutional amendment has been a way of avoiding that truth.

The other flaw in the whole enterprise is that, in appropriate circumstances, there is a need for governments to run a deficit. Keynes may not now be a congressional idol, but his arithmetic is impeccable. And there is no question that a government stuck in a recession when its rivals are not had better be ready to print a stack of currency notes, whatever the consequences. To deny a government that opportunity is to rob it of competence. That is what would have happened if the amendment had passed last week. □

Too-modest ambitions

The British government should not be content that 30 per cent of young people enter higher education.

THE British government has created another muddle for itself on participation in higher education. Last week, the higher education funding councils that channel public funds to universities and colleges made public a near-freeze in what they have to spend on the grounds that the proportions of young people going on to higher education have reached 30 per cent, which is the government's ambition. Ironically, it later emerged that the numbers of mature students now following courses at tertiary institutions, which are recorded separately, has greatly increased, so the participation rate substantially exceeds the magic 30 per cent.

There are two things to say about these developments. First, it is a creditable achievement that Britain has been able so to enlarge the proportions of the population in higher education, which have almost doubled in 15 years or thereabouts. The good news is that, in the past few years, there have been welcome signs that the expansion has been sustained by demand, the wish of students to win a higher or professional education. The decision five years ago to designate the old polytechnics as universities (much derided, but not by this journal, at the time) also appears to have had a beneficial influence. The bad news is that British academics remain more badly paid than those elsewhere, and that many institutions have never had the resources to conduct their academic affairs with style.

The second truth about last week's paradox is that the supposition that a participation rate of 30 per cent is a sensible target for British higher education. For one thing, the number is less than the participation rate in Japan (which hovers around 50 per cent). It is also less than current practice in France, where roughly 40 per cent of young people find their way into higher education. Why should Britain be so different, and so much less ambitious? Especially when it is now clear that the poverty of Britain's economic performance since the Second World War is largely attributable to chronic shortages of sophisticated skill, and not only in fields in which technical qualifications are necessary. The way in which mature students are flocking into British institutions is a sign that they, at least, appreciate the need. □

European Parliament rejects bid to stem confusion over gene patents

London. Attempts to lay down clear guidelines on whether human genes and transgenic animals can be patented have been rejected by the European Parliament. Last week, the parliament refused by 240 votes to 188, with 23 abstentions, to approve draft legislation aimed at tightening rules on patenting biotechnology products in member states of the European Union (EU).

As a result, the fate of applications for such patents will remain subject to the interpretation of the European Patent Convention (EPC), to which almost all EU states are signatories. In principle, this allows for patents on both genes and animals. But the convention is ambiguous, as it explicitly excludes patents on animal (and plant) varieties, and on any invention considered to threaten *l'ordre publique*, loosely translated as public morality.

Europe's biotechnology industry had seen the process of harmonizing patent legislation between the 15 EU member states as an opportunity to reduce such ambiguity. They point out that the convention was signed in 1973, before the full commercial significance of genetic engineering became apparent.

They also point out that, in principle, the failure of a seven-year effort to achieve this goal is unlikely in itself to have a major impact. Indeed, after a series of compromises aimed at reaching a single text capable of securing the necessary political support, an increasing number of companies were beginning to feel that they would be better off if the whole process was dropped.

In practice, however, many senior figures in the biotechnology industry fear that the outcome of the parliamentary vote will increase public pressure for greater regulation — and undermine efforts to reduce such regulation, which they claim is holding back European biotechnology in its competition with the United States and Japan.

"The outcome was appalling," says Peter

Doyle, an executive director of Zeneca Ltd, and vice-chairman of the Senior Advisory Group Biotechnology to the Federation of European Chemical Industries. "It is a very negative signal to the world at large about the worthwhileness of investing in biotechnology in Europe."

Indeed, Doyle describes last week's vote — the first in which the parliament has used

about the ethics of patenting life-forms.

"This decision will have a multiplier effect, and will certainly have an impact on our work; it is a great victory," says Jeremy Rifkin of the Foundation for Economic Trends in Washington DC, which is planning to launch a new campaign next month, backed by prominent community leaders, against patents on animals and human organisms.

Under the rules of the Maastricht Treaty, the parliamentary veto of a compromise text — agreed in January between its own representatives and those from the Council of Ministers — means that the whole attempt to harmonize national legislation on biotechnology patents between EU member states has been aborted.

The Commission will now have to decide whether to begin again from scratch. But industry became increasingly lukewarm about the draft directive as various revisions were introduced to meet the parliament's concerns about ethics. At the same time, decisions by the European Patent Office (EPO) have frequently gone in industry's favour, and many now doubt whether a new directive would be helpful.

A further effect of the vote will be to shift the focus of controversy back to debates on individual applications passing through the EPO. "The front will now move to where it should have been all the time," says Linda Bullard, a lobbyist with the Greens in Brussels who campaigned heavily against the compromise text.

According to Bullard, three particular cases will now become the target of legal protests: the granting of a patent to the Howard Florey Institute covering the gene for the production of relaxin, to the US seed company W. R. A. Grace on the products of the Indian neem tree, and to Harvard University and the Dupont chemical company on the so-called oncomouse.

In each case, the parliamentary vote may play a significant part in swaying the EPO's decision on whether a particular application contravenes *l'ordre publique*. Last month, for example, it rejected this argument in turning down an appeal against the relaxin patent.

A separate appeal against the oncomouse patent is being mounted by animal welfare groups such as Compassion in World Farming and the British Union Against Vivisection, whose own lobbying helped to defeat last week's proposed legislation. This appeal will be heard by the EPO in November, and is being widely seen as a test case for other patent applications on transgenic animals.

David Dickson

IMAGE
UNAVAILABLE
FOR COPYRIGHT
REASONS



New targets: activists are planning challenges to patents on the neem tree (above) and Harvard's 'oncomouse' (left).

its new powers under the Maastricht Treaty agreed in 1992 to veto legislation approved by the EU's principal intergovernmental body, the Council of Ministers — as an "indictment" of the parliament's powers. Giving in to pressure groups "is not democracy", he says.

But while the biotechnology industry is licking its wounds, the decision has given a political boost to pressure groups that have been using the debate about patenting to express criticism of genetic engineering and the way that it is used.

Many of them say that the vote's impact will not be confined to Brussels, but will provide support for anti-patenting campaigns across the world, ranging from farmers' groups in India protesting against the implications of the intellectual property provisions of the General Agreement on Tariffs and Trade, to debates in the US Congress

CERN asks Japan for support to build LHC

Tokyo. Christopher Llewellyn Smith, director-general of the European Laboratory for Particle Physics (CERN) in Geneva, Switzerland, last week officially asked Japan's minister of education, Kaoru Yosano, for his country's cooperation in the construction of CERN's planned Large Hadron Collider (LHC).

The move follows numerous unofficial approaches by CERN officials dating back to June 1992 (see *Nature* 357, 429; 1992). The ministry has said that it will discuss

Llewellyn Smith's request before deciding whether to ask for funding in the budget request for fiscal year 1996, due at the end of August.

After his meeting with Japanese officials, Llewellyn Smith declined to comment on the negotiations beyond saying they were "positive", and that they had covered both Japan's potential contribution to LHC construction costs and the possibility that it might be given associate membership to CERN.

David Swinbanks

France re-focuses nuclear research body . . .

Paris. France's Atomic Energy Commission (CEA) last week signed a four-year contract with the government formally endorsing the commission's withdrawal from state-funded participation in the development of new industrial technologies — an initiative actively promoted by previous governments — and its renewed focus on long-term research, particularly that relevant to the nuclear industry.

The government intends to establish these contracts with all state-funded research organizations, defining both their objectives and the means to achieve them, as part of the development of a national research strategy. It signed the first contract earlier this year with the computing agency INRIA.

The new contract between CEA and the government ends a decade of uncertainty about the role of the commission in French research, given the decreased research needs of the nuclear industry. "Our role in meeting the research priorities fixed by the government is now completely clarified," says Yannick d'Escatha, deputy chairman of CEA, who adds that guaranteed funding levels for the four years of the contract will provide welcome stability.

The contract was signed by the ministers for higher education, industry and finance, and reaffirms nuclear research as the CEA's major activity. But it adds that the commis-

sion should act as a supplier to meet the demands of its customers, namely the state, the nuclear industry and small and medium-sized companies in nuclear and other high-technology fields.

The contract also formally endorses the commission's role in other areas where it has acquired competence, such as integrated circuits and particle physics. For example, under a national strategy for life sciences due to be announced this week, CEA is expected to be given responsibility for coordinating radiobiology research among all state-funded bodies. It is also leading European efforts to build the superconducting magnets for the planned Large Hadron Collider (LHC).

Under the terms of the new contract, state funding for CEA's civil activities — FF6.2 billion (US\$ 1.23 billion) this year — will increase at the rate of inflation. This provides protection against budget cuts. But it also means that the CEA will either have to turn to industrial contracts, or sell some of the shares it holds in nuclear companies, if it is to find the money for a ten-year plan to boost investment in large research equipment, says deputy chairman d'Escatha.

The research priorities listed in the contract were hammered out during two years of negotiations between the government and CEA, and followed an extensive internal restructuring of the commission (see *Nature*

368, 6; 1993). Fields earmarked for increased support include research into the safety and performance of the next generation of fission reactors, funding for which will rise by 60 per cent during the four years of the contract.

The FF350 million that CEA now spends annually on research into the use of plutonium fuel will also increase significantly, with funding of research on mixed uranium/plutonium (MOX) fuel increasing by 80 per cent, and that on the incineration of excess plutonium stocks by 50 per cent. While current reactors can only burn 30 per cent MOX, future reactors will use the fuel at full strength, says d'Escatha.

Research on the separation and incineration of waste will also be increased by 50 per cent from FF650 million, while funding of the laser enrichment of uranium will be maintained at FF450 million a year. "We can't be behind in this area", says d'Escatha.

CEA will find the money for these priorities by withdrawing from all state-funded participation in industrial activities, and by reducing staff numbers. These have fallen from 23,000 to 18,000 over the past five years, and may fall by as much again. CEA says it hopes to avoid redundancies, perhaps by keeping recruitment rates lower than retirement levels. The government has said it will also transfer some CEA staff to universities.

Declan Butler

Research reactors may give Scottish reprocessing plant

Munich. The US Department and the UK Atomic Energy Authority (UKAEA) have been discussing the possibility of reprocessing in Britain highly enriched uranium (HEU) provided to non-US research reactors. The discussions suggest that the United States may be prepared to relax its strict policy requiring fuel rods containing HEU to be returned.

In particular, the United States has expressed interest in using the UKAEA's nuclear reprocessing plant at Dounreay, in the north of Scotland. Such a move could avoid the threatened closure of Dounreay's reprocessing plant, known as D1204, and provide it with ten more years of life.

Even if no such agreement is reached, the European Atomic Energy Agency (Euratom) and representatives of several European research reactors are already negotiating directly with Dounreay about reprocessing spent fuel rods, fearing that the United States may not be able to fulfil a previous promise to take them back.

As part of a 1978 agreement designed to stop commercial trading in weapons-grade uranium, the United States promised in 1978 to take back spent fuel rods of US origin from foreign research reactors if the lat-

ter agreed to convert from low-enriched uranium (LEU) to HEU, despite the relatively high costs of conversion.

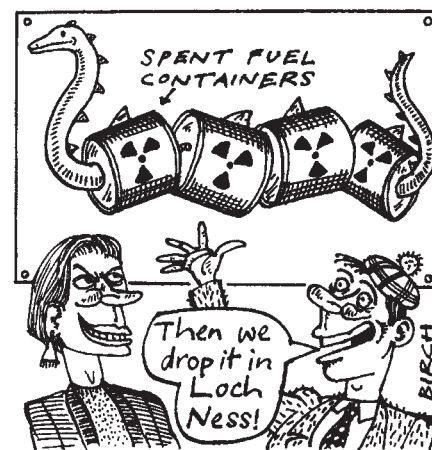
Last year, however, the first such shipment of fuel was initially refused entry into South Carolina, where it was intended to be stored at the Savannah River site, because of local protests (see *Nature* 371, 192; 1994).

The remaining waste — an estimated 15,000 spent fuel rods — is stretching dangerously the capacity of some research reactors' own storage facilities, but cannot be shipped to the United States until the Department of Energy (DoE) has completed a full environmental impact statement.

Under US law, such a statement must present all the possible options for handling the waste. One would be for Dounreay to reprocess at least some of the spent fuel. But under the 1978 agreement that could be done only if certain conditions were met.

First, the Dounreay plant would have to convert any HEU fuel it receives to LEU. Second it would have to refuse to handle fuel from reactors that are not willing to change over to LEU. Finally, it would have to agree to build an LEU reprocessing plant to encourage reactor conversion.

The talks between the US State Depart-



ment and the UKAEA have so far been inconclusive. The United States has been trying to persuade Dounreay to accept its conditions, arguing that an agreement could save the plant from closure. The UKAEA is said to be less than enthusiastic. But an official says that "if there is sufficient customer interest" it would consider building facilities to reprocess advanced LEU fuels.

The US National Control Institute (NCI), an independent group that monitors potential breaches of the 1978 agreement, is

... as UK agency cuts staff to prepare for privatization

London. Plans by the British government to privatize AEA Technology, the science and engineering division of the United Kingdom Atomic Energy Authority (UKAEA), took the next step forward last week with the publication of the Atomic Energy Authority Bill.

No decisions have been taken on the precise form that the privatization will take. "The bill deliberately leaves a number of options open so that we can make decisions nearer the time of sale," says Richard Page, the parliamentary under secretary of state at the Department of Trade and Industry.

In welcoming the bill, Sir Anthony Cleaver, the chief executive of AEA Technology said it will give the company freedom to expand its commercial activities.

But the move is being disputed by labour unions representing UKAEA staff, because they say it will lead to further cuts in research and technical staff that have already dramatically reduced the scientific capabilities of what has been Britain's main nuclear research and development organization since 1954.

Last year, the UKAEA, was formerly split into two as part of a long-promised privatization strategy. The UKAEA Government Division, will remain a government authority for nuclear facilities. The other, AEA

Technology, is being spun off as a general science and engineering service business. It now has an annual turnover of £250 million (US \$406 million), and made a profit of £10 million last year but only about half of its revenue comes from the nuclear industry through competitive tendering for decommissioning and safety contracts. The rest comes from general high technology consultancy and development work.

The government division holds approximately £8 billion of assets, which include nuclear and other facilities at Dounreay, Windscale, Risley, Harwell, Culham and Winfrith. It will remain responsible for sites, nuclear facilities, and awarding decommissioning contracts to companies including AEA Technology.

Nuclear research will not disappear completely. The agency still provides between £12 and £13 million a year for fusion research at Culham, for example, and works closely with the Joint European Torus project. But when this is compared with budgets of around £200 million in the late eighties, it is obvious some research has "fallen through the cracks" says Dr Derek Pooley, chief executive of the government division.

The British government argues that as the nuclear industry is now fully mature, research funding should be allocated on strictly commercial principles. "There are very strong differences of opinion whether what we have done in Britain is right or not," says Pooley.

But labour unions such as the Institute of Professionals, Managers and Scientists (IPMS) claim that moves to privatize AEA Technology undermines long-term planning in the industry. "We do not agree that the market should determine the research because it is very difficult to plan any longer than a few years," says Tony Wickett, Branch Secretary for IPMS.

Nick Parsons, head of media relations for AEA Technology, says the company's research budget is comparable to that of its competitors, five per cent of its annual turnover, or about £12.5 million.

The union is concerned about the other effect of government and the nuclear industry reducing research spend, namely staff numbers have been reduced since the 1980s from 14,000 to a total of just over 7000. The government division is not expecting any more redundancies but AEA Technology admits it is seeking 300-400 redundancies before the start of the next financial year (1 April 1995). "It is always a matter of regret but the only future for our business and staff is if we can be competitive," says Parsons. "It is essential to our survival, whether or not we are privatized."

Fiona Gammie

Russia links higher science funding to structural reforms

Moscow. The Russian cabinet has agreed to set up a new, streamlined Commission on Science Policy, headed by the prime minister, Viktor Chernomyrdin. The 20-member commission will be responsible for identifying research priorities.

The decision was taken a week after President Boris Yeltsin, in his annual address to the Russian Parliament, had admitted that public funds allocated to science "are insufficient to maintain the current level and amount of fundamental research". He urged a "structural reform of the whole scientific community".

In an subsequent address to the cabinet, the Minister for Science and Technology Policy, Boris Saltykov, emphasized that the government has cut

funding for science by 80 per cent over the past four years, leading to a drop of one-third in the number of scientists employed.

In some fields, he said, the losses are irreparable. But one of the key reasons for the current crisis was the delay in implementing structural reforms. Although promising to increase funding to three per cent of the total federal budget in 1996, he said that this should be accompanied by the closure of certain research centres, and greater efforts by the research community to adapt to the conditions of the market.

Indeed, the day after the cabinet meeting, the state Duma (parliament), in discussing the 1995 budget for the third time, agreed to approve a significant increase in funding of science. Their decision took the form of an amendment proposing to raise the research budget by a further 1,700 billion rubles from its current planned level of 5,400 billion rubles (US\$1.17 billion) — and to take this money out of government administration.

But Saltykov subsequently dismissed the proposal as a "crazy idea". Andrei Zakharov, head of the office responsible for the Committee for Education, Culture and Science, expressed his "horror" at the Duma's move. "Naturally I want Russian science to be healthy — but not at the price of destroying the state machine."

The state Duma is expected to take a final vote on the research budget tomorrow (10 March). It is widely expected to reject the calls for an extra 1,700 billion rubles — but to approve an increase of 300 billion rubles on the figures proposed by the government.

Carl Levitin

new lease of life

opposed to any reprocessing at Dounreay on the grounds that allowing reprocessing outside the United States would weaken its anti-proliferation policy.

But the talks are far from any likely action. The DoE will include the "Dounreay option" in its environmental impact statement. But it will not necessarily be accepted as the preferred option for dealing with HEU waste from research reactors. Even if agreement on reprocessing is reached with Dounreay, it is unlikely to take effect until 1996, by when various research reactors may have found solutions of their own.

Indeed, some reactors have now started talking to Dounreay independently about reprocessing their spent HEU fuel as they already have acute storage problems, and may be unable wait for the United States to make a firm decision. Dounreay has been encouraging such discussions because its D1204 plant, previously used for handling waste from fast breeder reactors, is threatened with decommissioning because of a lack of contracts.

The DoE wants to prevent the research reactors from negotiating directly with the UKAEA, without meeting its own conditions.

Alison Abbott

Concern raised over US plan for A-bomb survivor studies

Tokyo. Japanese and US researchers working for a foundation established nearly 50 years ago to study the effects of radiation on atomic bomb survivors are worried that a plan by the US Department of Energy (DoE) to change the management structure of the programme will jeopardize the autonomy of the foundation and put a further squeeze on its budget.

The Radiation Effects Research Foundation (RERF) in Hiroshima, and its predecessor the Atomic Bomb Casualty Commission have, since 1947, been studying under the management of the US National Academy of Sciences (NAS) the effects on survivors of the atomic bombs dropped on Hiroshima and Nagasaki.

The results of the study have been widely used in setting international standards for safe doses of radiation in the nuclear power industry, and the expertise developed by the foundation has been called on in other circumstances, such as the Chernobyl accident.

In January, the DoE dismayed RERF officials by saying that it wants to transfer the management of the programme from the academy to a US university to encourage radiation research in US universities as a whole. Columbia University in New York is a prime candidate for the job, but the department plans to ask for bids from other contractors. NAS, however, will not be allowed to participate.

Despite assurances from DoE officials that the new contractor's relationship with RERF will be unchanged from that with NAS, officials at the foundation foresee several dramatic changes in the reorganization.

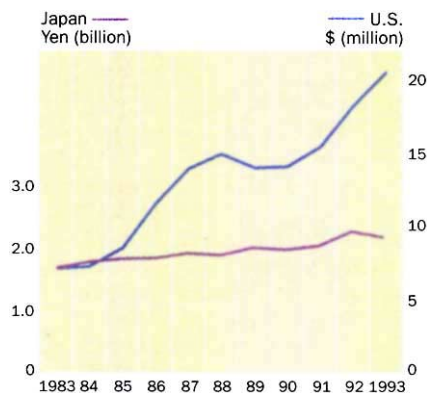
First, under a proposal already prepared by Columbia University, the university would not only play a management role and recruit staff but would also supervise the scientific content of the programme. In particular, it plans to create a computer centre parallel to that of RERF, to share RERF data and collaborate in research. Furthermore, the university would carry out assessments for DoE on the foundation's activities, for example comparing the value of atomic bomb studies to that of other types of research in radiation biology.

Itsuzo Shigematsu, the chairman of the foundation, says that there are potential conflicts of interest in the many roles suggested by Columbia. He and other RERF officials are concerned that, in the process, the neutrality of RERF may be lost.

"The A-bomb survivors and local governments of Hiroshima and Nagasaki have always been staunch advocates of the objectivity of studies at RERF," says Shigematsu. "From the very beginning, the reputation of NAS management for non-governmental

neutrality and scientific excellence became a symbol of the US determination to avoid real or apparent interference and to maximize the value of studies." He says that the NAS provides a "crucial buffer" between RERF and the US government.

Such concern is shared by many Japanese researchers involved in radiation research. Hiromichi Matsudaira, president of the Research Development Corporation of Japan and former director of the National



Despite equal contributions in yen, the US budget for RERF in dollars has escalated.

Institute of Radiological Sciences, says there is "no way" the DoE's plan can provide the scientific independence and autonomy of RERF which is "vital" for maintaining radiation protection standards and understanding the long-term effects of radiation.

Shigematsu says the DoE has never explicitly said why it wants to change the management arrangements. He and US scientists at RERF fear that the department's goal is to gain tighter control of the foundation and trim its budget, already being squeezed by the rising value of the yen.

Since it was set up in 1975, RERF has been funded 50:50 by the United States and Japan under an intergovernmental agreement. But over the past ten years, the value of the yen has more than doubled relative to the dollar. Thus, although the foundation's budget in yen has risen very little, the US contribution in dollar terms has tripled to \$23 million in 1994.

Under the resulting squeeze, the foundation has made severe cuts to hold down costs. The number of staff has dropped from 503 in 1983 to 375, while the number of US researchers has been cut from 22 to 11.

DoE officials say that they want to stimulate a declining interest in research on radiation in US universities by encouraging young researchers to enter this field. Both US and Japanese researchers at RERF fear that this may be at the expense of US researchers in Hiroshima.

David Swinbanks

House passes bill on cost analysis of new regulations

Washington. The US House of Representatives has approved a risk assessment and cost-benefit analysis act that would require formal risk assessment before federal agencies introduce any new regulation — an issue close to the heart of its new Republican majority.

Critics of the proposed legislation, including both the Clinton administration and the environmental movement, claim that the process of assessing risks on factors such as cancer risks suggested requires quantitative data in a form science is unable to provide (see *Nature* 373, 180; 1995).

But despite early talk of a compromise, no amendments were approved on the floor of the House during a debate last week, and the bill was passed virtually unchanged from a proposal published in January.

In particular, Republicans defeated a Democrat bid to prevent industries affected by a new regulation from being allowed to sit on the scientific peer-review panel considering that regulation. Amendments to prevent the peer-review process being challenged in court also failed to win support, causing Tim Roemer (Democrat, Indiana) to brand the proposal "the full employment bill for lawyers and lobbyists".

The fate of the measure now rests with the Senate. Its majority leader Robert Dole (Republican, Kansas) is a strong supporter of regulatory reform of the type proposed by the House. But John Chafee (Republican, Rhode Island), chairman of the Environment and Public Works Committee, believes the proposal would be a setback to efforts to protect the natural environment.

Colin Macilwain

Blood warning 'urgent' says Canadian judge

Quebec. The head of a public inquiry into Canada's blood supply system has called on hospitals to warn 3.5 million people who received blood transfusions in the late 1970s that they may be at risk of contracting not only HIV but also hepatitis C.

In an interim report, which recommends sweeping changes in the way that blood is collected and used, Mr Justice Horace Krevier said that neither public health authorities nor the Canadian Red Cross Society had adequately informed transfusion recipients about the risks of testing.

He proposed that mandatory informed consent should be obtained from all potential transfusion recipients, except in emergencies, and that more use should be made of alternatives, such as autologous blood donations.

David Spurgeon

NASA debates 'privatizing' its space centres

Washington. Officials at the US National Aeronautics and Space Administration (NASA), faced with shrinking budgets and the prospect of laying off government scientists, are considering transferring some work to 'privatized' research institutes run by universities or other outside organizations.

Although the idea is still in its preliminary stages, NASA managers are planning to start talking to universities and consortia this week about the possibilities.

One approach would be to create institutes affiliated with particular NASA centres. Stanford University and the University of California, for example, already have ties with the Ames Research Center near San Francisco and might be interested in closer cooperation. Similarly, the Lunar and Planetary Institute, located adjacent to the Johnson Space Center outside Houston, Texas might expand its charter to become a centre for human exploration of space, incorporating both planetary science and life sciences.

Another option would be to look for a single organization — perhaps a consortium such as the Universities Space Research Association — that could manage the entire network of affiliated research centres "to give it more cohesion", says France Cordova, chief scientist at the space agency.

Many NASA scientists would welcome such a scheme. Most could probably continue working at their current centres, merely being paid from a university rather than directly from the government. But what they would gain in freedom from bureaucracy might be offset by the insecurity of living on government contracts.

The idea of privatizing research at the centres is discussed in a 17-page draft report by Cordova and the heads of NASA's three offices responsible for space science, micro-gravity and life sciences, and Earth science. The report was written in response one released last month by an internal NASA review panel, set up by administrator Dan Goldin and called the 'Red Team', that was intended to provoke debate on how the agency should be restructured.

Although the science managers say the new report "should in no way be construed as an official NASA 'science plan,'" they take issue with some of the Red Team's suggestions and float several ideas of their own "to enlarge the dialogue".

Science at Ames could be privatized, they say, using as a model the Institutes of Geophysics and Planetary Physics (IGPP), operated at multiple sites by the University of California. They suggest that the Goddard Space Flight Center in suburban Washington DC should reduce its science managers and contractor scientists. And that the National Oceanic and Atmospheric Administration, which operates US weather satellites, might take over responsibility for data

distribution from NASA's proposed Earth Observing System.

Beyond such specific recommendations, the draft report challenges certain basic premises of the Red Team report, such as the idea that considerable overlap exists in the science conducted at different NASA centres. "Redundant naming does not necessarily imply redundant capability," it says.

They point out, for example, that even though Johnson, Ames and the Jet Propulsion Laboratory (JPL) all do "planetary science", Ames focuses on planetary biology, Johnson on planetary materials and samples, and JPL on planetary geophysics and atmospheres.

The Red Team task force, which did not include any scientists, argued that each centre's mission should be narrowly focused. In contrast, the science officials support diversity and multidisciplinary research, and the conduct of certain types of research at more than one NASA location.

While the Red Team propose giving JPL responsibility for all basic space science — not counting Earth science, which would go to Goddard — the science managers would amend that to "primary responsibility", and then only for planetary science.

JPL, owned and operated by the California Institute of Technology, is generally considered a good model for privatization. But giving JPL overall responsibility for NASA-financed science programmes could create its own problems.

Last week, for example, the agency announced the winners of a competition for low-cost planetary missions in the 'Discovery' series. A Lockheed-Ames concept for a cut-rate (\$59 million) mission to map the Moon and search for ice at its poles, dubbed the Lunar Prospector, was approved for launch in 1997. (Three more missions — to

return cometary dust samples, to study the Venusian atmosphere, and to collect solar wind particles and return them to Earth — were picked for further study.)

Although JPL scientists and engineers were involved in many of the 28 proposed Discovery concepts, NASA intends to award contracts equally to NASA centres, universities and private companies. If JPL managed the Discovery programme, it would have an inherent conflict of interest.

Even though the NASA science officials claim that privatization would offer benefits — and presumably savings — their report argues strongly that the agency should still maintain a core of staff scientists. "Credibility of internal science expertise, both basic and applied, is important to provide maximum support for external guest investigators and to assure NASA's critical capacity to act as a 'smart buyer'," they write.

Privatization would bring many uncertainties, not least whether it really would save money. Many agency scientists question whether JPL operates more cheaply than other centres. And NASA is not even certain how much money it needs to save.

Wesley Huntress, head of the office of space science, predicts that the spending power of his office will decline by 18 per cent between now and 2000. But that takes into account only cuts already made by President Bill Clinton to finance his proposed tax cut for the middle class. Congress has yet to suggest its own cuts.

But some kind of reorganization is inevitable. Goldin, has told his staff to brace for significant reductions in personnel, to be decided in a series of reviews over the next two months. Huntress told an advisory committee for Solar System science last week, that "this administrator is going to change the centres, period."

Tony Reichhardt

White House aide to quit policy office

Washington. M. R. C. Greenwood (right), associate director for science at the White House Office of Science and Technology Policy (OSTP), is to leave the administration on 1 May to return to California to care for a friend who has breast cancer.

An accomplished researcher into the genetic causes of obesity, Greenwood is regarded in the science community as an effective liaison with the White House. She says she is "very sorry to be leaving".

As the only associate director at OSTP responsible for basic science — previous administrations had two, one for life sciences and one for physical sciences — Greenwood has had a tough job fighting basic research's corner within a adminis-



tration orientated to technology.

But she successfully pushed through the administration's science policy document, Science in the National Interest, last August, and claims some of the credit for maintain-

ing research funding level in Clinton's 1996 budget proposal. "I think we have a very respectable 1996 budget," she says. In May, Greenwood will return to her previous job as dean of graduate studies at the University of California at Davis.

Colin Macilwain

Clinton team trims laboratory reform plans

Raleigh, North Carolina. Plans by the Clinton administration to streamline the federal laboratory system — including those run by the Department of Energy, the Department of Defense (DoD) and the National Aeronautics and Space Administration (NASA) — are in trouble after separate reviews of each of the three agencies failed to produce coherent proposals for change.

Katherine Gillman, staff director of the interagency review that is being carried out by the White House Office of Science and Technology Policy (OSTP), says that finding overlaps between the reviews is turning out to be difficult. "Maybe what we recommend to the President will be more specific [to each agency] than we had anticipated," she said last week.

Roche asked for more *Taq* patent evidence

London. The Swiss pharmaceutical company Hoffman-la Roche has been told by the European Patent Office (EPO) that it needs to carry out further experiments to confirm the difference between its *Taq* polymerase and similar thermostable enzymes isolated by previous researchers from the same organism before the EPO is prepared to grant the company a patent.

In doing so, the patent office has gone back on a previous decision — communicated to the company last August — indicating that it had agreed to issue the patent on the enzyme (see *Nature* 372, 212; 1994). Rights to both the enzyme and the polymerase chain reaction (PCR) in which it is used were bought by Roche from the US biotechnology company Cetus in 1991 for the sum of \$300 million.

Shortly before the patent was due to be granted in December, the EPO received objections from three different sources. The identity of the objectors has not been revealed. But each is believed to have made arguments similar to those put forward by the company Promega in its US challenge to the patent, suggesting that the enzyme 'discovered' by Cetus scientists was identical to that described several times previously in the literature (see *Nature* 373, 310; 1995).

As a result, the EPO has now decided to take the unusual step of reopening the examination procedure. In particular it has asked Roche to provide further experimental data "to proof [sic] the 'high fidelity' of the claim polymerases in comparison to the polymerases of the state of the art".

This is a reference to a crucial claim in the Roche application, namely that the novelty of its *Taq* is proved by its significantly greater accuracy in reproducing nucleotide sequences compared to the polymerases that had already been isolated by others. □

OSTP is due to report on the future of the government's \$15 billion-a-year laboratory system in April. Its report is supposed to be based on reviews requested from the three agencies. An external review of the Department of Energy laboratories was completed last month by a panel chaired by Bob Galvin of Motorola (see *Nature* 373, 463; 1995), and internal reviews were delivered last week by DoD and NASA.

The DoD review suggests that the armed services should continue to run their own laboratories, but contains few concrete proposals for reform. NASA's document calls for each of its laboratories to focus their activities more tightly (see page 107).

Gillman told the annual meeting of the scientific society Sigma Xi at Raleigh, North Carolina, that neither report addressed the central question of how the size of the laboratories can be reduced — as budget projections require — without a civil service-style attrition process that would freeze hiring and cause the best people to leave.

She added that the DoD has tested a new

system of hiring and firing civil servants at the China Park base in California which might form the basis for laboratory reform. Meanwhile Galvin has put forward radical proposals to 'corporatize' energy department laboratories; but these may prove to be politically unacceptable.

"The governance problem at the Department of Energy is something that we're going to have to fix," says Gillman. "The laboratories cannot live with the existing system — it is wrecking them." But she added that OSTP could not promise to include firm proposals for the management of the energy laboratories in its long awaited April report.

In fact, the interagency review, which was initiated by Clinton's OSTP as one of its top priorities, has been overshadowed by last November's election result. The White House may now let the Republican majority in Congress take the lead in reforming the federal laboratories, as such reform is bound to be unpopular — at least in the short term.

Colin Macilwain

Breakaway iceberg 'due to warming'

IMAGE
UNAVAILABLE
FOR COPYRIGHT
REASONS

London. The breaking away of large expanses of ice from the eastern side of the Larsen ice shelf supports the idea that recent ice loss from this region of the Antarctic ice sheets is linked to regional atmospheric warming rather than ocean currents, according to scientists from the British Antarctic Survey (BAS). "It is real confirmation that it is atmospheric changes [that are responsible]," says David Vaughan, a glaciologist with the BAS at Cambridge.

The first image taken by one of five NOAA polar orbiting weather satellites (above left) shows the Antarctic Peninsula (60°S, 60°W) on 9 January this year. The far right-hand corner of the image is dominated by loose sea ice up to a black line running along the 60°W longitude,

which is an area of open water just in front of the ice shelf. The ice sheet is marked by striations caused by melt water and a mountain ridge.

Five and a half weeks later (16 February) a 2,400 km² iceberg, roughly the size of Oxfordshire, had broken away from the ice shelf. This can still be seen on the image (above right) taken on 27 February, which also shows that another section of the ice shelf has broken up into ice rubble.

"We think these represent the second and third break-up of the ice shelf in this area," says Vaughan. The first caused considerable debate on whether the break-up of the ice sheet could be used as a measure of climate change (see *Nature* 350, 328; 1991). Fiona Gammie

Minister's visit gives boost to UK/South Africa science links

Cape Town. As part of a move to strengthen renewed scientific links between Britain and South Africa, Ben Ngubane, South Africa's Minister of Arts, Culture, Science and Technology, has been invited by the British government to deliver the third Zuckerman Memorial Lecture in London in October.

The lectures are named after Solly (later Lord) Zuckerman, the Cape Town-born zoologist who went to the University of Oxford in 1934, and later rose to become chief scientific adviser to the British government (1960–66).

The invitation came at the end of a visit to South Africa last week by a British delegation headed by David Hunt, the science minister, which included meetings on biomedical science at the University of Cape Town and on agricultural research at the University of Stellenbosch.

Describing biotechnology as the key to the dual challenge facing South African agriculture — namely meeting the requirements of both national planning and the abolition of trade tariffs required by General Agreement on Tariffs and Trade — Sakkie Pretorius, professor of microbiology at Stellenbosch, said that “co-operative research with Britain could help us significantly”.

Hunt's visit has been widely welcomed in the scientific community. Friedel Sellschop, co-chairman of Ngubane's advisory committee, emphasized its particular significance for South Africa's planned ‘technology foresight’ exercise. This will be conducted with British assistance, and a South African representative will be seconded to the Office of Science and Technology in London to participate in an assessment of the current British technology foresight exercise.

South African scientists are also enthusiastic about the decision of the two governments to set up of a modest fund to finance joint projects between research teams in the their respective countries. But they warn that the money needs to be used carefully. “If properly used, this money should unlock other sources of funding”, says Sellschop. “But very careful consideration should be given as to how it is spent. In particular, small-scale projects should be encouraged.”

Michael Cherry



Ngubane: to give London lecture.

University blocks efforts to reveal researchers' identity

San Francisco. The University of California has successfully resisted demands that it should reveal the identity of scholars researching library documents on the health effects of smoking that are claimed to have been stolen.

The ruling was made last week by a Superior Court in San Francisco as part of a battle between the university and the tobacco company Brown & Williamson over ownership of the papers. The documents suggest that the company concealed data for decades about the addictive nature of smoking.

“B&W's panic regarding the documents is not surprising,” UC attorneys wrote in a memorandum to the court in February. They described the contents of the documents as of “immense public and academic importance to smoking and public health and the history of tobacco regulation”.

But the company claims they contain confidential client-attorney communications, as well as attorneys' notes and sensitive trade secrets. It says that some of the documents were improperly released by a congressional subcommittee, and that others were stolen by an assistant paralegal who had worked on a project for the company.

The materials were sent anonymously to Stanton Glantz, a professor of medicine at the University of California who is an expert on smoking and public health. He passed them to the library and put together a team of researchers to study the material — a project that the university told the court was critical to academic and public knowledge.

Brown & Williamson has now asked the court to instruct the university to return the papers. It also wants the university to release library records that would show who had

provided the documents and who had studied them, arguing that it has to take steps to prevent further releases of the material.

According to the university, the company was so keen to check who consulted the documents that at one point it sent private detectives to sit outside the library archives and note everyone who entered.

The university told the court that it considered the documents were now in the public domain, and that Brown & Williamson's conduct suggested that the company was willing to go to unacceptable extremes to stop research it believed to be unfavourable.

Glantz praised the university's efforts to defend the documents and researchers' privacy. “The University of California is taking a very principled interest in academic freedom and the public interest,” he said.

UC is just one of many institutions to receive copies of the Brown & Williamson documents. The US media has reported extensively on their contents, and they have been debated in congressional hearings. The tobacco company attempted to demand inspection of copies held by members of Congress and the media, but the US District Court for Columbia quashed the subpoenas.

In delivering an opinion on the latter case, Judge Harold H. Greene said that the tobacco company was trying to intimidate and harass those attempting to bring to light evidence that it may have concealed knowledge that its products were both health hazards and addictive.

The San Francisco court is to hold a hearing on 17 March to resolve the issue of access to the documents. At present, this is limited to lawyers for both sides of the case.

Sally Lehrman

Molecular studies centre planned for Bonn

Munich. Science is to benefit from the post-reunification decision to reinstate Berlin as the capital of Germany. As part of a compensation programme to Bonn, the federal research ministry and the research ministry in Nordrhein-Westfalen, the regional authority (*Land*) that covers the city, have agreed to set up a foundation to support the creation of an ambitious new interdisciplinary research centre in the Bonn area.

The foundation will be established with DM750 million (US\$523.5 million). DM685 million will come from a federal compensation fund, and the *Land* intends to make up the balance. Part of the capital will be used to build new laboratories for the centre; conversion of one of the existing office buildings, many of which will be left empty when the government leaves has been ruled out

on safety grounds.

The centre is to be known as the Centre of Advanced European Studies and Research (Caesar). It will avoid the lack of flexibility from which many Germany research institutes suffer by employing nearly all of its 300 scientists — who will work in 60 small groups — on limited term contracts of at most ten years.

The research groups will work on complex molecular systems that demand interdisciplinary approaches, for example neural networks or optoelectronics. The Caesar centre is intended to compete with research establishments such as the Princeton Institute for Advanced Studies in New Jersey and the Scripps Research Institute in California. A committee will be established to draw up detailed procedures in the next few weeks.

Alison Abbott

UK withdrawal from INTEGRAL

SIR — The United Kingdom's withdrawal from the European Space Agency (ESA)'s INTEGRAL mission (*Nature* 373, 459; 1995) is not just a story of the offended interests of some British scientists: it is a tale of Britain turning its back on a European agreement, leaving fellow ESA member states to pick up the pieces. Although playing the role of *prima inter pares*, Britain did not "initially propose" INTEGRAL, as stated in the article. Rather, it was a European, and indeed worldwide, collaboration, to propose the INTERNATIONAL Gamma Ray Astrophysical Laboratory.

Indeed, to describe the instrument, proposed by A. J. Dean of the University of Southampton as principal investigator, as a "UK/Italian project" neglects the important French contribution, as well as the Spanish and Finnish/Norwegian/Swedish participation in that proposal.

As to the background, INTEGRAL was selected by ESA's science programme committee in June 1993 as its next 'M' (medium class) mission. Britain voted in favour; to quote from the minutes: "The UK delegation thoroughly approves the recommendation of INTEGRAL, which had come at a particularly good time". A few weeks earlier, there had been a public debate at the Royal Institution in London on UK involvement in the next ESA science mission. INTEGRAL was selected from a number of candidates, partly on the strength of the proposed contribution to the payload by British groups. Costs were of course discussed. Indeed, aware of the potential problems, Roger Bonnet, director of ESA's space science programmes, had asked for particular accuracy and transparency in this exercise. The UK national delegates were therefore fully aware of the cost implications of the payload when voting so enthusiastically in favour of INTEGRAL.

Britain's recent decision to withdraw therefore appears to be the result of a misunderstanding (or management blunder) between scientists who put forward a mission in good faith and a financing body that is now reneging on an agreement with the rest of Europe. Perhaps the really interesting story is the one behind this spectacular change in the UK position.

There is an interesting precedent. About 25 years ago, Britain withdrew from an earlier European science mission, again a gamma-ray mission (COS-B) in which the University of Southampton was to have played a key role. The result was a damaging blow for many years to gamma-ray astronomy in Britain and a very successful mission for the rest of Europe. *Historia magistra vitae?*

As to picking up the pieces, it is now obvious that the other ESA nations will

have to do their part. Italy is cautiously trying to do so, essentially because INTEGRAL exists, not because of "economic logic". Italy and others are trying to save a mission crucial to ESA's science programme that has been put in grave danger by Britain's withdrawal.

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SIR — Your article on the ESA science programme and the United Kingdom's difficulties in participating in INTEGRAL, the medium-class mission approved last year, raises some important issues for the future of space science in the United Kingdom. This mission was originally proposed from the United Kingdom and the UK team played a leading role in the studies that led to its selection against severe competition. Technology development has taken place in the United Kingdom over a 10-year period in preparation for this mission and it is not surprising in consequence that it was rated top priority here for the mission selection and that the UK delegation to the ESA science programme committee then voted accordingly last year. The likely costs of UK participation were well known and have not changed substantially.

If UK scientists cannot, with this degree of careful preparation, participate in the ESA programme, one wonders in what circumstances they will be able to do so. Ken Pounds, the chief executive of the Particle Physics and Astronomy Research Council (PPARC), has offered the explanation that his new research council inherited a good programme but an inadequate budget from its predecessor, the Science and Engineering Research Council. From his point of view that is no doubt all there is to say, but for those of us who expected to help build and launch the INTEGRAL instruments it is not convincing, nor should it be for those who hope to play similar roles in the future missions in ESA's programme. The UK companies that should have had a major part in the design and construction of advanced gamma-ray and optical instrumentation have reason to feel particularly dismayed. The funding of the domestic space programme has for a number of years been subject to recurrent crises resulting from a combination of the long-term nature of the programme and the failure of the research councils to provide secure planning for funding on the necessary timescale. It is essential that PPARC, in conjunction with the British National Space Centre, which has the responsibility for national planning in space matters, should find a solution to this problem.

The United Kingdom will, in any case,

contribute at significant cost to the INTEGRAL mission through its subscription to the ESA science programme. At issue here is the return to UK astronomy through access to the observations INTEGRAL will make and through the stimulation of a healthy UK gamma-ray community.

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Common noun

SIR — Hi-jacking names may be more common and more aggressive today than most of us think (*Nature* 373, 370; 1995). In Germany we have another shining example. The pet food company Effem GmbH, Verden/Aller has registered the word 'Pedigree' as a trademark. It is used to market food for dogs. The word 'pedigree' meaning the ancestry of an individual is widely used among geneticists, and thousands of articles have been published using this word. A nice feature of the noun is that it is identical in several languages, for example English, Spanish, French, and German. Furthermore, the word is used not only by the scientific community but also by practical breeders of any kind. More stringent rules should apply when trademarks are registered.

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Keep off politics

SIR — The Smithsonian wouldn't have got itself into this mess if it had stuck to its last (*Nature* 373, 371; 1995).

By all means debate the Enola Gay and the dropping of the A bomb, but not at the Smithsonian's Air and Space Museum: it is an entirely inappropriate place to do so. This Mecca to aviation is the world's supreme technological showplace and the crown jewel of the Washington tourist scene; it has never been previously tarnished with contentious political messages attached to an exhibition and nor should it be now.

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Impact of behavioural ecology

SIR — To be a complete scientist one must publish the results of research. So scientists (as well as granting councils, job search committees and university administrators) use publication records as a measure of a scientist's worth. Publication, however, provides only the raw material for scientific progress — the value of a publication can also be assessed, at least in part, by evaluating citations. Indeed, an entire industry has grown up around such citation analysis^{1,2}.

Journals are also subject to citation analysis as a measure of their influence on science^{2,3} and *Nature* and *Science* are at the top of the multidisciplinary sciences category². But imagine my surprise when the newest journal (*Behavioral Ecology*) in my own field (behavioural ecology) rocketed to the top in the most recent compilation in *Journal Citation Reports*² (*JCR*). *Behavioral Ecology* began life in 1990 as the official organ of the newly formed International Society of Behavioral Ecology and rapidly attracted good papers. Even so, in this, its first inclusion in *JCR*, it was ranked number one (based on impact factor) among the 30 journals in the behavioural sciences category (including psychology and neurobiology) and number two among the 96 zoology journals — a remarkable feat for a newcomer to any field.

Unfortunately, the *JCR* analysis² is incorrect. The accompanying figure tells the story. The meteoric rise of *BE* in *JCR* has come at the expense of *Behavioral Ecology and Sociobiology* (*BES*), long among the top 2 or 3 journals in both the behavioural sciences and zoology categories as compiled by *JCR*. Of the 116 papers

published in *BES*, 28 and 29 were cited 224 times, but 200 of these citations were erroneously attributed to *BE*. The corrected figures put *BES* back among the top 10 journals of both the behavioural sciences and zoology categories in *JCR*.

Clearly the similarity in the titles of these two journals leads to human error (which will probably be blamed on computers) in the compilation of citations. Other pairs of journals with similar titles in the *JCR* listing may also suffer from errors of this kind.

Behavioural ecology is a vibrant field with a notable impact on medicine⁴, psychology⁵, evolutionary biology⁶ and sociology⁷, so it is not surprising that it can foster two first-rate journals such as *BE* and *BES*. Both journals deserve continued support. But publications about these publications sometimes need more careful scrutiny.

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1. Garfield, E. *SCI Science Citation Index 1993* (Institute for Scientific Information, Philadelphia, 1994).
2. Garfield, E. *SCI Journal Citation Reports; a bibliometric analysis of journals in the 1993 ISI Database* (Institute for Scientific Information, Philadelphia, 1994).
3. Garfield, E. *Science* **178**, 471–479 (1972).
4. Williams, G. C. & Nesse, R. M. *Quart. Rev. Biol.* **66**, 1–22 (1991).
5. Blurton-Jones, N. G. *Ethol. Sociobiol.* **11**, 353–359 (1990).
6. Andersson, M. *Sexual Selection* (Princeton Univ. Press, Princeton, 1994).
7. Daly, M. & Wilson, M. *Homicide* (A. de Gruyter, New York, 1988).

Quote/misquote?

SIR — In the article "Warning of retaliation over access to physics facilities" (*Nature* **371**, 728; 1994), I do not recognize the quotation attributed to me: "Given the way the costs of major facilities are going in this country, there won't be a spallation source built in the United States, so we'll need to share facilities"; it neither reflects my opinion nor is it anything I could have stated with certainty. The US neutron science community has expended considerable effort in developing a consensus position on future neutron sources as represented in the report *Neutron Sources for America's Future* prepared for the US Department of Energy by a panel of experts from the neutron science community. The two primary recommendations from this report state:

Recommendation 1: Complete the design and construction of the ANS [Advanced Neutron Source] according to the schedule proposed by the project.

Recommendation 2: Immediately authorize the development of competitive

proposals for the cost effective design and construction of a 1-MW pulsed spallation source. Evaluation of these proposals should be done as soon as possible, leading to a construction timetable that does not interfere with rapid completion of the ANS.

I totally support these recommendations.

At present, the United States and Europe support both steady-state research reactors and accelerator-based spallation sources because they are complementary sources of neutrons necessary for science and industry, and both will be needed for the future.

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■ Our reporter's shorthand note of the conversation, when transcribed, reads as follows:

"Given the way the costs of major facilities are going in this country there won't be a spallation source built in the US and a neutron source in Europe, so we will need to share facilities.

"We [would] provide access to ANS for European colleagues in return for us getting access to ESS [European Spallation Source]."

Editor, *Nature*

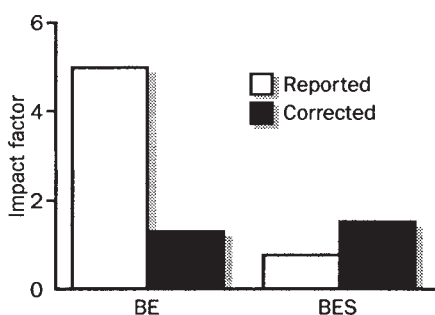
Advice to contributors

If you've got a thought that's happy,
Boil it down.
Make it short and crisp and snappy,
Boil it down.
When your brain its coin has minted,
Down the page your pen has sprinted,
If you want your effort printed,
Boil it down.

Take out every surplus letter,
Boil it down.
Fewer syllables the better,
Boil it down.
Make your meaning plain. Express it
so we'll know, not merely guess it;
Then my friend ere you address it,
Boil it down.

Cut out all the extra trimmings,
Boil it down.
Skim it well, then skim the skimmings,
Boil it down.
When you're sure 'twould be a sin to
Cut another sentence into,
send it on, and we'll begin to,
BOIL IT DOWN!

Anonymous. From *Treasure Trove*,
p.115, London 1924



Reported¹ and corrected impact factors (IF) for *Behavioral Ecology and Sociobiology* (*BES*) and *Behavioral Ecology* (*BE*) for 1993. IF is the average number of citations in a given year for papers published in the previous 2 years. I used ref. 2 to determine corrected IF values for 1993 by tabulating citations (correctly or incorrectly attributed to these journals) for each paper published in 1991 and 1992. This method undoubtedly misses some citations, especially when authors names are misspelled, but such errors should be random with respect to journal.

Letters submitted for Correspondence should be typed, double-spaced, on one side of the paper only.

Universities waste science funds

Peter Williams

Universities in Britain are unable to cope with the demands of the modern research enterprise. But who will ensure that the necessary changes to the system are made?

UNIVERSITIES in Britain receive more than £1 billion each year in grants for research from many sources. This money is nearly all given after a time-consuming, competitive application procedure involving peer-reviewers and adjudicating committees. When an award is made, its administration is undertaken in the university where the grant-holder works.

British universities, in general, control the funds centrally, pay personal stipends through their payrolls and delegate to individual departments the handling of the expenses element. Departments expect grant-holders to make their own purchases and pass the invoices to the departmental administration to check and transmit to the university's central office. The purchases are once more checked, invoices paid and reimbursement claimed from the donor — a cumbersome and time-wasting process. Considerable amounts can be lost if bulk purchasing arrangements, unpopular with individual researchers, are not used. In addition, departments need to keep inventories, and be alert to deadlines for reports and closing dates for renewals and submission of accounts.

There are also significant problems for university research in handling commercial opportunities. A system for making contracts with commercial companies is needed, using high-quality legal advice. If patentable discoveries are made, arrangements must be made for their registration and for the payment of the considerable costs involved; future protection must also be arranged. Without a satisfactory system, the university may lose a great deal of potential income.

Need for change

In parallel with these requirements for handling a grant system, there is the question of the distribution of the overhead payments received from the research councils and, in the case of the charities, through the Higher Education Funding Council. If these overheads do not support the research that earned them, something has gone wrong.

Finally, the filtrate from all this university activity reaches the donor organization, which has to process and pay out funds from the grants it has awarded. The donors between them have about 45,000 current grants at any one time. My experience as the director of a donor organization and working with a university depart-

ment with a large grant income has led me to believe that there is a need for a drastic change. Let me therefore give a few examples.

Recipients of grants are asked to submit claims 3 months in arrears. Who is expected to carry the costs of this delay? One major charity that awards fellowships pays the first year's stipend in advance but requires a report before it sends out the second instalment. That charity has accumulated a bank balance of £10 million because the reports have still not been supplied, yet universities are still paying the fellows' stipends! In many cases, cheques are sent out just before Christmas in the knowledge that they will not be deposited for 10 days.

In one large university, the processing of information from the grant-holder through his or her department to the central administration is conducted through two or three computer systems with a print-out each month of 5 kg of spreadsheet. A centralized system has been developed at Cambridge, but other university computer systems do not have the sophistication to handle the workload. It is not yet possible to buy a computer program that will handle the day-to-day needs of a large department. A program could be devised, at some cost, but has not been, presumably because such a task falls between the needs of large industry and small businesses.

The lack of cash-flow management in a university can be very expensive. Taking the example of Cambridge (the figures are similar for Oxford), the university spent £75 million from more than 3,000 grants last year. Half was paid in arrears, which cost the university more than £500,000 in lost interest. On a national scale, the sum wasted must have been more than £8 million. This sum could be saved if the research councils and charities paid up on time, which most of them would be willing to do if the situation was explained to them and the requisite data supplied.

At the level of the individual researcher, an immense amount of time is spent in writing applications, submitting reports and the piecemeal ordering of research materials. This time away from the bench could be lessened with the use of modern electronic systems that transmit information to and from the supplier, university administrator and funder. But, above all, scientific competition and the potential financial value of university research re-

quires sophisticated administration and speed of action. If universities are to operate in this field, they need entrepreneurial management, decentralization, greater flexibility, sound financial planning and modern technical capacity.

No perfect model is available, but the research laboratories of industry and business-efficiency advisers have the expertise to produce suitable systems. Universities will have to accept that the money available for scientific research has outstripped their capacity to cope, under the present arrangements. More is required than a tinkering with the present bureaucratic, committee-bound, precedent-governed system, which is too slow for the research world of today.

Is it too much to hope that modern methods of information transfer and accounting technology should be applied to university-based science? Or has this not been done because funding for research comes from too many sources, each with its own rules? If that is the case, surely there must be a role for the Department for Science, the Association of Medical Research Charities and other organizations to collaborate to make access to their funds less cumbersome and reimbursement less complex. Or are there too many universities, each operating a separate budget within an independent framework and unable to afford to change to new systems and modern technology? If that is the case, there seems to be a role for, say, the Committee of Vice-Chancellors and Principals. It is difficult to accept the plea of poverty in the presence of waste caused by inefficiency.

The need for change is urgent. The methods can easily be developed. The cost relative to present wastage is minimal. The result would produce a much less frustrating and time-wasting situation than research endures today. The proliferation of grant funding to the universities and the monetary value of scientific achievement now need a response from the universities receiving the grants to show that they have the capacity to handle the large and growing sums flowing in. The alternative is for the funds to be used elsewhere. □

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The top quark found at long last

The announcement from Fermilab last week is neither a mere formality nor the capstone of a theoretical edifice, but another landmark in the long road to telling how matter is constructed.

THE announcement at Fermilab that two detectors catching decay products from particles formed in proton-antiproton collisions have finally provided clinching evidence that the 'top' quark exists has an unavoidable sense of anticlimax. People have been talking about it for a long time, after all. And given the way in which the quark explanation of nuclear matter has proved over the years to have predictive power, what more natural than that its existence should be taken for granted?

But that would be mistaken. Science hangs on the empirical discovery of what the real world is like. However solid the theoretical basis of some field of inquiry may be, and however confident people may be about its predictions, experimental verification does more than confirm people in their prejudices. Often, verification is a dramatic reminder of the poverty of human imagination.

So much is clear from the history of the idea of neutron stars. The idea that a sufficiently massive star that had exhausted its thermonuclear fuel might collapse into a mass of neutrons appears first to have been suggested by Baade and Zwicky in 1935. Their natural conclusion was that such an object would be relatively dull, a massive object emitting no energy worth talking of and thus virtually undetectable. But real-life neutron stars, when discovered in 1969 as pulsating stars, have proved to be among the most spectacular and interesting objects in the Galaxy. Who predicted that that would be the case?

The top quark (called 't') is potentially a much simpler entity. The theorists, building on Murray Gell-Mann's original idea, say that there should be six quarks, with the whimsical names of 'up', 'down', 'strange', 'charm', 'bottom' and 'top', or u, d, s, c, b and t. Their function is to constitute the more familiar particles of nuclear matter. A proton consists of two u's and one d, for example, a π^+ meson of one u and an anti-d.

Because, like electrons, quarks have half-integral spin, each quark has an anti-particle *doppelgänger*, with identical mass, but opposite electric charge. There is now even more than a hint that the number of quark 'generations' (the pairs u and d, s and c, b and t) must be limited to three, but that hangs on the assumption that the three known forms of neutrino have no mass, which may be false. Otherwise, the discovery of t seems neatly to fill out the list of the expected quarks. Why should

not physics now turn to simpler things?

First, there is the business of the mass of the top quark reported last week. One group, that responsible for the D0 detector, reports a mass equivalent to 199 GeV; the other, a mass equivalent to 176 GeV. (The two estimates are consistent with each other, given that the first group quotes statistical and systematic errors of ± 20 and ± 22 respectively, and the second group, ± 8 and ± 10 .) These values are much greater than expected only a few years ago, implying that each t is some 200,000 times more massive than a proton, and more than 10 million times as massive as a u. In themselves, they explain why the top quark has been so long waiting in the wings. The beams of protons and antiprotons from the Tevatron at Fermilab have been generating particles with an energy equivalent to 1.8 TeV (10 per cent below the maximum design energy) for months now, and there are still only a few handfuls of significant events to boast about.

All the other quarks are much less massive. The lightest is u, with a mass of a few MeV, next comes d, between 1.5 and 4 times as massive, then s (more than 100 MeV), c (about 1.5 GeV) and b (about 5 GeV). Why should t be up to twenty times more massive than its partner? Nobody seems very clear. Nor is there any very clear explanation of the 25-fold mass difference between s and its partner, c.

Indeed, there is bound to be ambiguity about what exactly is meant by the mass of a single quark, when quarks seem not to exist except in combinations with others. The Fermilab measurements are essentially measurements of the mass of the meson-like entity $\bar{t}$, the combination of top and antitop.

The implicit assumption is that the mass of each quark is half that of the combination. But quarks are supposed to be held together by gluons, while a certain amount of binding energy must be involved. Half the mass of the $\bar{t}$ pair may bear very little relation to the mass of a bare quark, or even to the values that the theoreticians should enter into the equations they have lovingly constructed. The hope is that all this will become plain when the Higgs particle is found.

Other issues are even more perplexing, and not least what is called the 'confinement' problem: why do not bare quarks exist? It may, of course, be relevant that quarks have electrical charges that are not

integral multiples of the unit electronic charge (d, s and b have charge $-\frac{1}{3}$, u, c and t have charge $+\frac{2}{3}$), and that the space-time in which we live will not accommodate non-integral charges, but that is simply a way of replacing one conundrum by another. "Why not?" is the natural and proper response.

Another aspect of that problem, the less serious restrictions there appear to be on the occurrence of quark composites, has however been accommodated by the invention of a novel qualitative property of all quarks: colour. Colour is a kind of analogue of electric charge, except that it comes in three forms ('red', 'green' and 'blue', of course). Like colours repel, unlike colours attract, so a composite of two quarks will hold together only if they are differently coloured, whereupon only a quark of the third colour can be added in a stable fashion to the composite.

The notion of colour is the source of the prefix in 'quantum chromodynamics', the field theory of nuclear matter. It is the analogue of the successful theory of quantum electrodynamics in this more difficult field. By common agreement, it is an awkward theory to calculate with, yet that has not prevented people from using it as a component in the formulation of the grand unified theories, called 'GUTs', which are sometimes expected to amount to the famous 'theory of everything'.

It is easy to make fun of this enterprise, but it is also unfair. We forget that Gell-Mann's first version of the quark model (when three rather than six quarks seemed sufficient) seemed in the late 1950s to be an over-imaginative and even artificial way of categorizing the particles then known. It became respectable after the prediction (by Gell-Mann in 1962) of a particle called Ω^- was verified. (The Ω^- is a composite of three s quarks which, because of their identity, cannot be in the same state and so must have spin $3 \times \frac{1}{2}$.)

Who can say that the same will not happen with the coloured-quark view now in vogue? The only certainty is that much more will have to be learned in the laboratory of how quarks behave before there is grist for the mill of speculation and prediction. And even then, there will be much deeper questions about the link between what nature allows and the structure of space-time crying out for attention. The discovery of the top quark is just another landmark in the long process of learning how matter is constructed. **John Maddox**

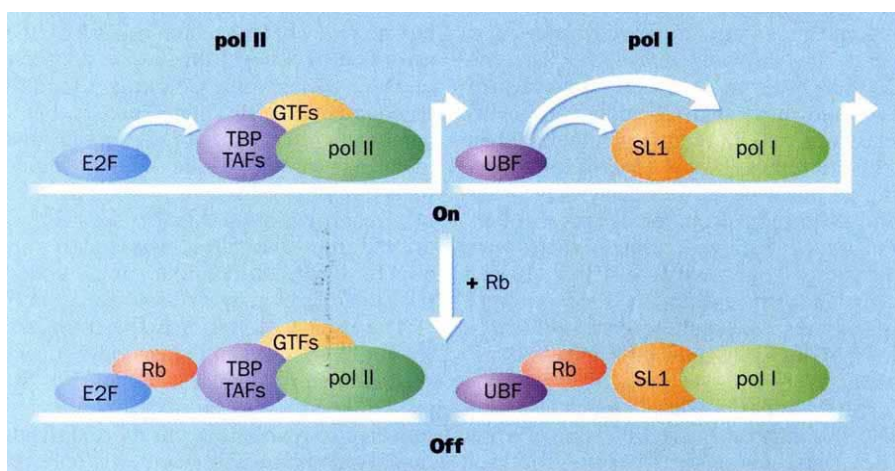
Pol I gets repressed

Brian D. Dynlacht

A KEY player in the study of the regulation of transcription during the cell cycle has been the retinoblastoma gene product Rb, the first tumour suppressor to be identified. The prevailing wisdom has been that Rb suppresses cell growth by preventing the expression of those genes, transcribed by RNA polymerase II (pol II), that are necessary for proliferation. The work of Cavanaugh *et al.*, described on page 177 of this issue¹, now suggests an additional mechanism for Rb-mediated growth suppression, in which Rb inhibits activation of transcription by RNA polymerase I (pol I) as well.

ribosomal RNA by pol I, shutting down the synthesis of these genes could be a most effective way for Rb to inhibit cell proliferation.

At least three components are necessary for maximal transcription of rDNA genes by pol I, including the promoter selectivity factor SL1, the upstream-binding factor UBF, and the polymerase itself²⁻⁵. UBF binds to the pol I promoter and, once bound, can interact directly with the polymerase⁶ and probably with SL1 as well. Cavanaugh *et al.* show that Rb specifically blocks activation of pol I transcription by UBF and that this inhibi-



Models for Rb repression of transcription from promoters for RNA polymerases II and I, illustrating similarities of mechanism. Rb can repress pol II transactivation mediated by E2F (curved arrow) or other upstream activators that interact with the basal transcriptional machinery, including TFIID (composed of TBP and TAFs) or other general transcription factors (GTFs). Repression of UBF-stimulated transcription from the pol I promoter by Rb requires binding to UBF, which may block interactions between this activator and SL1 or pol I, or both. In both cases, the end result is transcriptional repression.

Previous studies examining the mechanism of growth suppression have focused on the ability of Rb to repress transactivation by the transcription factor E2F, a factor implicated in the expression of genes required for entry into the DNA-synthesis (S) phase of the cell cycle (reviewed in ref. 2). This repression is abrogated by phosphorylation of Rb, which renders the protein incapable of associating with important targets. There are many cellular proteins apart from E2F that can bind to unphosphorylated or hypophosphorylated Rb, and most of them are sequence-specific transcription factors that are associated with a proliferative signal.

It might be expected that a global repression of genes would be necessary to arrest cell growth, and the work of Cavanaugh *et al.* suggests another mechanism for Rb repression which could readily effect such global changes. As growing cells depend on the steady synthesis of

tion requires an interaction (presumably direct) between UBF and Rb. The authors demonstrate this association in two ways *in vitro* and furthermore show that the UBF-Rb complex is also present in cell extracts.

These results are satisfyingly consistent with previous findings⁷, in which a cDNA encoding UBF was isolated on the basis of an expression library screen using purified Rb protein as probe. Moreover, the biochemical data of Cavanaugh *et al.* are supported by studies at the cellular level: in these experiments, the monocyte-like cell line, U937, can be induced to differentiate by the phorbol ester TPA, which causes an accumulation of Rb in nucleoli, the site of rDNA transcription. Concomitant with this accumulation, there was a marked decrease in rRNA synthesis, while the amount of Rb associated with UBF increased.

The new work points to further experiments that should extend our under-

standing of the threads that tie together transcriptional activation of different polymerases, and provide further insight into how repressors such as Rb function (see figure). Interesting analogies already spring to mind between the ways in which Rb might repress transcription by pol I and pol II. For example, stimulation of pol II promoters by upstream activators, such as E2F, may be blocked by preventing this factor from interacting with components of the basal transcription machinery, such as the TATA-box-binding protein (TBP) and TBP-associated factors (TAFs). Similarly, association of Rb with UBF may block the effect of this activator on pol I or SL1, itself composed of TBP and pol-I-specific TAFs⁸.

The results of Cavanaugh *et al.* open many avenues for exploration. For example, we should find out whether repression of pol I transcription by Rb is a result of an inability of UBF-Rb complexes to bind DNA or of interference with interactions between UBF and pol I (and possibly SL1). A mutational analysis will be required to determine whether the association between UBF and Rb is mediated through a region of UBF overlapping the transcriptional activation sequence, or through the segment in the third HMG-box repeat containing an LxCxE motif, a sequence found in several viral and cellular proteins that interact with Rb. Also, the effect of phosphorylation of Rb on the repression of UBF can be readily tested. It will be interesting to see if Rb can mediate repression of transcription by RNA polymerase III as well, especially in light of the finding that UBF can associate not only with pol I but also with a subunit of yeast pol III (ref. 6).

Despite the pace of progress over the past five years, a number of questions remain regarding Rb function. Many of these can be addressed using rigorous biochemistry now that several important transcriptional targets of Rb have been identified. Finally, another finding of Cavanaugh *et al.* that merits further investigation is the possible role of Rb in the differentiation of haematopoietic cells. □

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Slab burial grounds

Thorne Lay

AFTER cooling at the Earth's surface for about 150 million years, the oldest regions of oceanic plates become buoyantly unstable and sink into the mantle, creating subduction zones. The sinking oceanic slabs are colder than the surrounding mantle, cold enough for frictional faulting or phase changes to cause earthquakes within them as far as 700 km down. Seismic waves propagate at relatively high velocities within the cold slab as well, and the three-dimensional spatial extent of high-velocity slab material, including any aseismic regions, can be determined using seismic tomography.

On page 154 of this issue¹, van der Hilst describes how he has used seismic information to determine the geometry of the oceanic slab descending under the Tonga–Kermadec island arc. His interpretation that the Pacific slab extends into the lower mantle to depths of at least 800 km is significant given that the geometry of mantle convection is controversial^{2,3}, but he also demonstrates that the tectonic history of the subduction zone influences the configuration of the deep slab.

Subduction zones have complex tectonic histories, progressing from a poorly understood initiation phase, to maturation commonly accompanied by opening of a back-arc basin and seaward migration of the trench, to cessation of convergence, often involving collision with a subduction-resistant oceanic plateau or continent. The fate of downwelling slabs must be considered in the context of the time-dependent evolution of the associated subduction zone, particularly if there is a rapid interval of trench migration. This is because the geometry of the slab is controlled by both the position of the slab's leading edge and the position of the trench (see figure). If a slab penetrates into a relatively high-viscosity lower man-

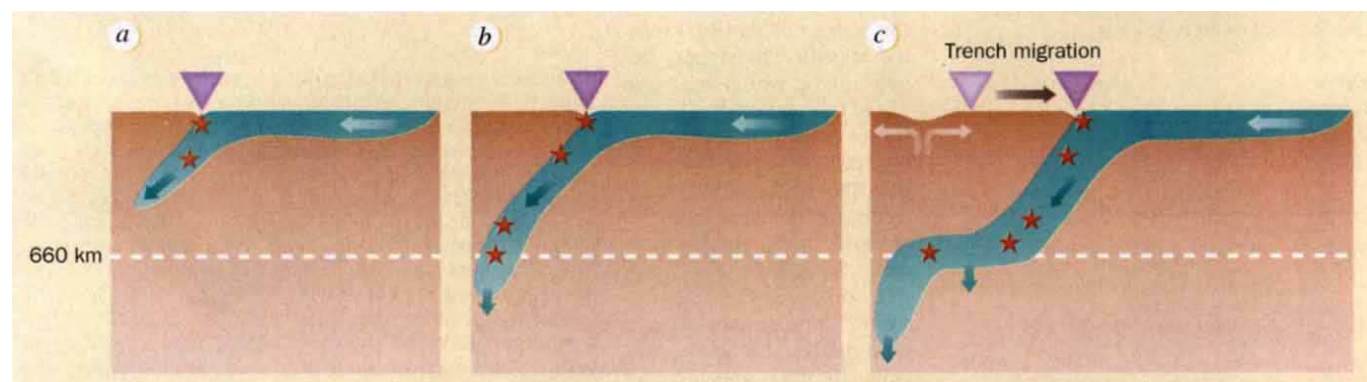
tle, its leading edge can effectively be pinned and any seaward trench migration must be accommodated by horizontal deflection⁴. Factors influencing slab geometry are the relative rates of slab sinking (controlled by the age of the subducted material and the vertical viscosity structure encountered) and trench migration (coupled to the onset and vigour of back-arc opening). Moreover, stresses induced by the ambient pattern of mantle flow can distort slab geometry, an effect believed to be responsible for several hundred kilometres of lateral shortening of the Tonga slab⁵.

For more than 18 years seismologists have been using large data sets of arrival times from earthquakes in subduction zones to create tomographic images of elastic wave velocities near subducting slabs⁶. Much attention has centred on the Tonga slab, which has the highest incidence of deep earthquakes and the fastest sinking velocity of all current downwellings (resulting in lower temperatures at a given depth than in other slabs). It only takes about 10 million years for a slab to sink to a depth of 700 km, and it is expected to remain thermally anomalous at that depth; indeed, the slab material warms up so slowly that detectable velocity heterogeneity may still exist for material that was subducted as long as 40 million years ago. Many subduction zones have consumed far greater lengths of slab than are illuminated by earthquake activity, and by imaging the seismic velocity structure near the maximum depth of seismicity, seismologists can attempt to resolve whether the aseismic portion of the slab extends into the lower mantle or is deflected horizontally and confined to an upper mantle layer.

Evidence for horizontal deflection of some slabs has been used to argue that the

mantle-convection system is strongly layered, with separate upper and lower mantle cells⁷; however, horizontal deflection alone cannot be used to resolve the slab's fate. All slabs are expected to encounter significant resistance to penetration of the lower mantle because of phase transformations in the slab materials around a depth of 660 km (ref. 8), and possibly because of an increase in lower mantle viscosity². The slab may buckle and fold over on itself, producing localized horizontal deflections, even while it continues to sink. Rapid trench migration combined with slow descent into the lower mantle could cause the slab to flatten out on the 660-km phase boundary between the upper and lower mantles, producing relatively extensive but ephemeral horizontal distortion. In such a case, the slab's lower-mantle extension may be significantly offset from the present-day zone of deepest seismicity.

The geometry of the Tonga–Kermadec slab is complex even in the seismically active region⁵, and one might expect its aseismic extension to be even more so. Reconstructing the tectonic history of the subduction zone over a 20–40-million-year interval is important because the place where slab material subducted long ago may be quite different from where the same slab is sinking today. van der Hilst's image of the northern Tonga arc, which has experienced considerable recent back-arc opening, has a horizontally deflected portion of slab connecting to an offset aseismic lower-mantle extension penetrating to at least 800–1,000 km. van der Hilst is able to image the entire slab by using events from several subduction zones, with arrival times for New Hebrides events helping to illuminate the offset deep-slab extension. Deep earthquakes under the Fiji plateau are distributed nearly horizontally at about 600 km, and van der Hilst's model indicates that the southern group of these events is located within the horizontal segment of the Tonga slab. Under Kermadec, a



A sinking ocean plate in various stages of evolution of a subduction zone. Earthquake locations in the slab are shown by stars. *a*, Initially, the slab penetrates with relatively little deformation as the leading edge sinks through the upper mantle. *b*, When the leading edge encounters the 660-km phase boundary, which resists penetration to

at least a certain extent, the slab may begin to buckle, with any lower-mantle extension sinking relatively slowly. *c*, In some subduction zones, the opening of a back-arc basin and seaward trench migration occur, which could result in the slab deflecting horizontally on the 660-km boundary.

region that has not experienced strong trench migration, the Pacific slab appears to penetrate steeply to depths of at least 800 km. Reconstructing the tectonic history of this region is difficult, however, and alternative models relate the anomalous deep earthquakes under Fiji to slab extensions from now-extinct subduction zones along the boundary between the Indian and Pacific plates⁹.

If rapid trench migration is actually associated with horizontally deflected deep slabs, one would expect to find other examples analogous to the northern Tonga situation. The best analogue is perhaps the Izu-Mariana subduction zone. The Mariana arc has undergone little trench migration and there is tomographic evidence for nearly vertical penetration of the Pacific plate to at least 800 km. The Izu region to the north has experienced significant eastward trench migration and, like Tonga, has anomalous deep earthquakes displaced westward from the main slab activity along with high seismic velocities extending horizontally towards the west at depths from 500 to 660 km. The region's back-arc spreading history and present-day distribution of seismicity, and seismic tomography images, also point to horizontal deflection of the deep slab¹⁰.

van der Hilst's images of the Tonga-Kermadec slab indicate some slab penetration into the lower mantle, along with deflection of that portion of the slab where rapid trench migration has occurred. But one must be cautious in interpreting the overall mantle convection configuration from such images. Even for a chemically layered mantle in which no mixing between upper- and lower-mantle cells occurs, a viscous downwelling slab is expected to depress the chemical boundary by at least several hundred kilometres. The 660-km seismic boundary is attributed to phase transitions that will inhibit, though probably not prevent, a slab from penetrating the lower mantle. But the existence of a chemical contrast, possibly at a greater depth, with a density contrast sufficient to induce convective stratification, has not been ruled out. □

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Ringing necks with dynamin

Regis B. Kelly

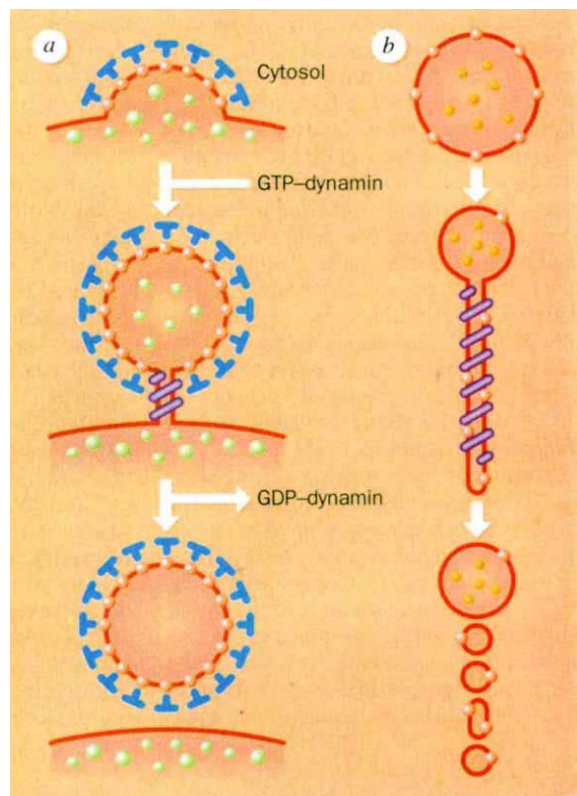
SYNAPTIC vesicles in nerve cells are packages of neurotransmitters that are ejected each time a neuron fires. The stock of vesicles is then replenished rapidly by endocytosis of their membrane components from the plasma membrane and repackaging with neurotransmitter once inside the cell. An agent instrumental in sustaining the supply of vesicles, dynamin, has now been collared, and the deft way in which it executes its task is described on pages 186 and 190 of this issue^{1,2}.

Dynamin, a large GTPase, is needed for the endocytosis of surface membrane in a large number of cell types, including neurons³. Hinshaw and Schmid² show that it can adopt a helical structure in solution; Takei *et al.*¹ demonstrate that it is this helical structure that enables dynamin to wrap around the neck of membrane vesicles and help pinch them off from the plasma membrane. Dynamin-like molecules do not seem to be universally required for membrane fission. An ability to self-assemble into helical arrays, however, may be a common feature of dynamin and its homologues and could explain their physiological properties.

Endocytosis of membrane proteins from the plasma membrane involves three steps⁴: clustering of the membrane proteins by clathrin and its associated proteins, invagination of the membrane into a constricted clathrin-coated pit and, finally, severing of the neck to give a coated vesicle (see figure). *In vitro*, this sequence can be arrested at different stages, depending on which analogue of GTP is present⁵. The defining characteristic of the constricted-vesicle stage is that the neck allows small molecules to pass into the vesicle, but excludes protein. Structures that look like constricted vesicles are found at the surface of mammalian and *Drosophila* cells in the presence of mutant forms of dynamin⁶⁻⁸. Although it seemed plausible that GTP hydrolysis by dynamin could be necessary for severing

the neck of the coated pit, it was not clear just how it did it.

Dynamin is abundant in brain (1.5 per cent of total protein), and possesses, in addition to a GTPase domain, a pleckstrin-homology domain, and a carboxy-terminal proline-rich domain containing an SH3-binding motif³. The demonstration of its ability to self-assemble into long helical structures in the test tube² is complemented by the elegant electron micrographs from De Camilli's laboratory¹, which show that dynamin surrounds the neck of the membrane (a in the figure). When permeabilized nerve terminals are incubated in the presence of the non-hydrolysable GTP analogue,



a, Model of dynamin function during endocytosis. A coated pit forms by the binding to membrane proteins (pink) of clathrin (blue) and associated proteins specialized for the uptake of neurotransmitter from the cytosol. Large and small molecules can enter the pit. In the second step, GTP-dynamin (purple) causes a constriction around the neck of the pit. Small molecules, but not proteins (green) can enter through the neck. If GTP hydrolysis is prevented, endocytosis arrests here. In the third stage, GTP-dynamin is hydrolysed to GDP-dynamin and the neck is severed. After membrane fission, no extracellular molecule can enter the vesicle. b, Model of how a dynamin analogue, the yeast VPS1 protein, might affect sorting. A spherical organelle containing soluble (yellow) and membrane proteins is induced to form a protrusion by a dynamin-like protein. Soluble proteins are excluded from the protrusion because of its narrow diameter. By vesiculation of the protrusion, small vesicles are generated that contain membrane protein but not soluble ones. Mutations in the dynamin-like protein disturb the sorting process.

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GTP- γ S, long invaginations are formed which are coated with spirals of dynamin (Fig. 1 of ref. 1). Clathrin-coated bulbs are often seen at the tips of the invaginations. The spiral of polymerized dynamin squeezes the membrane tube to an outside diameter of about 30 nm, and an internal diameter tight enough to prevent the passage of proteins (*a* in the figure). The GTP-associated form of dynamin is believed to ring the neck of the constricted pit^{1,2}: GTP hydrolysis could change the configuration of the helix, squeezing the neck further until membrane fission occurs in a sort of molecular garrotting.

This pinching-off of membrane necks occurs frequently in biology, in events as diverse as cell division, budding of membrane-coated viruses, and vesicle budding from an intracellular organelle. If a molecule like dynamin is required in each case, then GTP hydrolysis should be necessary for membrane fission (*a* in the figure). This is not true for budding from the endoplasmic reticulum or Golgi, where GTP hydrolysis is required for vesicle uncoating and docking but not for vesicle formation. Dynamin may be able to help bud small vesicles from the plasma membrane but not from intracellular membranes because of the difficulty of curving a stiffer membrane. Plasma membrane differs fundamentally from intracellular membranes in that it is supported on the inside by cortical cytoskeleton and is attached to matrix and other cells on the outside.

Homologues of dynamin all have highly similar GTPase domains. One class of analogues, the Mx proteins, can confer resistance to viral infection. Until now it has been difficult to see a functional link between dynamin and homologues that regulate viral infection. An exciting clue comes from the observation that the mouse Mx protein can also polymerize into C-shaped and helical molecules, depending on the nucleotide concentration, that are 11 nm thick and 100–150 nm long, parameters similar to those found for dynamin-1 (ref. 9). Thus the dynamin family may share a propensity to form

filamentous aggregates, regulated like microtubules by GTP hydrolysis. Perhaps Mx proteins interfere with virus infection by wrapping around cylindrical structures of about 25 nm diameter.

There is also an intriguing link between dynamin and VPS1 protein, a dynamin-like protein in yeast involved in protein sorting. Mutations in VPS1 send vacuolar proteases to the surface and a Golgi enzyme to the vacuole^{10,11}. Most models of protein sorting require a mechanism for removing membrane proteins from an organelle, leaving behind the soluble protein content. Such segregation is needed, for example, to return low-density lipoprotein (LDL) receptors from the endosome to the cell surface, after the LDL particles themselves have been internalized for the purposes of removing their cholesterol cargo. The canonical model (*b* in the figure) is that a membrane protrusion forms, thereby excluding soluble proteins. Considering dynamin's remarkable ability to constrict tubes, it is feasible that a dynamin analogue such as

the VPS1 protein could facilitate membrane protrusion (and therefore membrane sorting) by a common mechanism. The VPS1 protein and dynamin-1 both have the 'self-assembly' domain essential for the polymerization of mouse Mx protein⁹, suggesting that VPS1 may also form helices. Hinshaw and Schmid's data, however, caution us that the proline-rich, SH3-binding, carboxy-terminal region, which is not conserved among dynamin homologues, is required for polymerization of dynamin *in vitro*.

Nonetheless, the roles of dynamin in membrane fission and of VPS1 in sorting probably both depend on the ability to constrict membrane tubes using helical polymers. If this turns out to be the case, then other mutations that affect sorting should involve dynamin-like proteins. □

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POLLUTION

Too much of a good thing

Peter D. Moore

ONE of the most insidious forms of pollution is eutrophication of natural habitats by airborne nitrogenous materials. This is an all-pervasive process; and the upshot of such aerial deposition of fixed nitrogen in the form of nitrates and ammonia may be serious modification of the overall nitrogen budget of nutrient-poor ecosystems. Of particular concern are the acid heathlands and calcareous grasslands of western Europe, the characteristic flora and fauna of which stem largely from their low-nitrogen soils. How are they being affected? In the latest issue of *Environmental Pollution*¹, Pitcairn and colleagues provide graphic evidence of increasing foliar concentrations of nitrogen in mosses and dwarf shrubs, and they point to the likelihood of changes in the make-up of the plant communities involved.

Much of Europe suffers from aerial deposition of fixed nitrogen derived from both natural and human sources. Oxides of nitrogen are formed during the combustion of fossil fuels and the burning of biomass, as well as by natural processes, such as lightning strikes and soil microbial activity. Ammonia is generated as a by-product of agriculture, especially in the generation and application of animal wastes. The intensification of both agricultural and industrial activities has led to increased deposition of nitrogen in these forms, especially over the past 20 years. From a level of about 2–6 kg N ha⁻¹ yr⁻¹

over much of Europe 20 years ago¹, some areas, such as oak–birch woodlands in the Netherlands, now receive 60 kg N ha⁻¹ yr⁻¹ or more².

To see whether these changes have been reflected in the uptake and hence the foliar nitrogen concentrations of plants, Pitcairn *et al.* have used herbarium specimens of mosses (mainly *Sphagnum* species) collected during the 1950s, 1960s, 1970s and also in 1989, and have analysed their leaf tissues. They have also analysed the foliar nitrogen content of heather (*Calluna vulgaris*) from a range of British sites and altitudes.

The moss studies reveal considerable geographical variability. The northwest of Scotland, for instance, shows no change over the past 40 years, but this is the area of the country least likely to be affected by increasing aerial deposition of nitrogen (current levels about 6 kg N ha⁻¹ yr⁻¹). Other parts of Scotland showed 38–45% increases in foliar nitrogen (current aerial input levels are 10–15 kg N ha⁻¹ yr⁻¹) among the species analysed. Cumbria, in northern England, displayed a 62% increase (current input levels 30 kg N ha⁻¹ yr⁻¹) during the 40-year period. So the increasing deposition of nitrogen is reflected in the uptake of this element by mosses. But in the case of these acid-loving species which inhabit nutrient-poor areas, such eutrophication is likely to prove supra-optimal and even inhibitory to their healthy growth.

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The foliar nitrogen content of *Calluna* was examined over a much wider area, including the heaths of southern England, and the highest values (2.6%) were found in the eastern part of lowland England where agriculture is most intensive. There is a simple correlation between nitrogen deposition at the sites sampled and the foliar nitrogen concentration in *Calluna*. From the slopes of these linear correlations, it can be estimated that an increase of 1 kg N ha⁻¹ yr⁻¹ results in an increased foliar concentration of 0.045 mg g⁻¹ dry weight.

Such an increase in leaf nitrogen makes a plant much more attractive to grazers, and one implication is that grazing animals (especially invertebrates) will become a greater problem. Increasing damage by heather beetles has in fact already been recorded. Perhaps more sinister is the advantage gained by plants with more aggressive growth rates as a consequence of the nitrogen enrichment of heathland. Some grass species, in particular, are replacing heather on the lowland heaths of the Netherlands. In Britain, it is not surprising that *Calluna* is declining on the East Anglian heaths and that *Sphagnum* species are failing to regenerate on the southern Pennines.

The effects of this form of widespread and occult pollution are difficult to detect and to control. Input of aerial nitrogen that has a detrimental influence on the growth of bog mosses may well have a stimulatory influence on other species. In Norway spruce, for example, the foliar nitrogen content is positively correlated with sulphur deposition from aerial pollution³ and this could act as a growth stimulant for this species. Indeed, enhanced growth of this sort could result in magnesium deficiency as the plant outgrows the availability of the element and thus produce the symptoms of the *Waldsterben* (forest death) syndrome⁴.

Meadow grasslands could also be affected. Silvertown *et al.*⁵ have analysed data from the Park Grass Experiment at Rothamsted Research Station, England, which has been running since the last century, and show that there is a positive correlation between rainfall and the first harvest of biomass each season over a 90-year period. The correlation is strongest in plots that had been treated with no extra nitrogen fertilizer, so it could be argued that in these sites the additional water input was also bringing

supplies of nitrogen and thus stimulating growth. Which all goes to show that one plant's poison may bring another plant prosperity.

In nitrogen-poor chalk grasslands, one such plant that may have benefited from the increasing aerial input of nitrogen is the invasive torgrass (*Brachypodium pinnatum*) which is proving a conservation headache over much of western Europe⁶. Many chalk grasslands, especially in the Netherlands and southeast England, are becoming dominated by this robust plant and are losing their former diversity. But a 20-year experiment by Wells⁷ in a south-

ern English grassland shows no significant change in community composition despite the rising input of aerial nitrogen. This may be a consequence of the continued grazing regime at the experimental site, which prevents more aggressive species assuming dominance. The maintenance of intensive management is evidently the best approach available to counteract the influence of the airborne bombardment of such habitats by nitrogen. □

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INTRACELLULAR RECEPTORS

New wrinkles in retinoids

Bogi Andersen and Michael G. Rosenfeld

FRESH insight into the mechanisms responsible for the spectacular effect of retinoids on organ development is gained from two papers, one by Saitou *et al.* on page 159 of this issue¹ and the other by Imakado *et al.* in *Genes and Development*². Both use targeted expression of a dominant negative retinoic acid receptor (RAR) in transgenic mice to investigate the stage- and organ-specific roles of retinoids in mammalian development. In contrast to the prevailing view that retinoids inhibit epidermal differentiation³, results from these experiments, in which dominant negative RARs are expressed in epidermis, suggest that retinoids may in fact promote normal epidermal maturation.

Retinoids act through two types of receptors, RAR and retinoid X receptors (RXR). Each receptor has three distinct isoforms (α , β and γ); alternative splicing of isoforms leads to further heterogeneity. Two bioactive retinoids that activate these receptors, all-*trans*- and 9-*cis*-retinoic acid, have been described. Retinoic acid receptors bind both ligands, whereas RXR only binds 9-*cis*-retinoic acid, suggesting that two distinct retinoid-response pathways exist in the cell⁴. Ultimately retinoids regulate cellular differentiation and proliferation by controlling gene expression: RARs and RXRs bind DNA sites consisting of repeated hexamer purine-G(G/T)TCA sequences. RXR binds as a homodimer to an element that consists of a direct repeat separated by one base pair (DR+1), whereas RAR appears to bind DNA as a heterodimer with RXR to DR+1, DR+2 and DR+5 elements⁵.

Epidermis is a stratified squamous epithelium composed of a single layer of proliferating basal cells and several layers of post-mitotic suprabasal cells. Basal cells express keratins 5 and 14, but as these cells move towards the skin's surface they

leave the cell cycle and switch to expression of keratins 1 and 10. During further differentiation and upward migration, these cells express proteins required for formation of a cell envelope and eventually undergo programmed cell death to form stratum corneum. This layer, the protective barrier between an organism and its environment, consists of the cell envelope linked on the inner surface to a keratin network and on the outer surface to a lipid multilamellar structure⁶.

Investigation of the role of retinoids in normal epidermal development *in vitro* has shown that vitamin A depletion promotes and retinoid treatment inhibits epidermal keratinocyte differentiation³. In contrast, a classical study on the effect of vitamin A deficiency on embryonic development revealed dramatic developmental abnormalities in several organs, yet there was no mention of defective skin formation⁷; these abnormalities are also seen in animals with targeted disruption of retinoid receptors⁸⁻¹¹. However, nutritional experiments cannot define the role of RAR in skin formation as they do not account for possible ligand-independent functions for RAR, nor do they consider incomplete retinoid deficiency.

To clarify the role of retinoids in organ formation, the technique of homologous recombination has been used to disrupt retinoid receptors in mice. This approach is complicated because several different isoforms of RAR and RXR exhibit both distinct and overlapping expression patterns during development, and in the adult. Disruption of individual RARs revealed functional redundancy for these receptors, suggesting that multiple receptors must be knocked out to disable completely the retinoid pathway in a given organ. Consistent with the possibility of redundancy is the finding that individual disruption of both RAR- α and RAR- γ genes has no effect on skin devel-

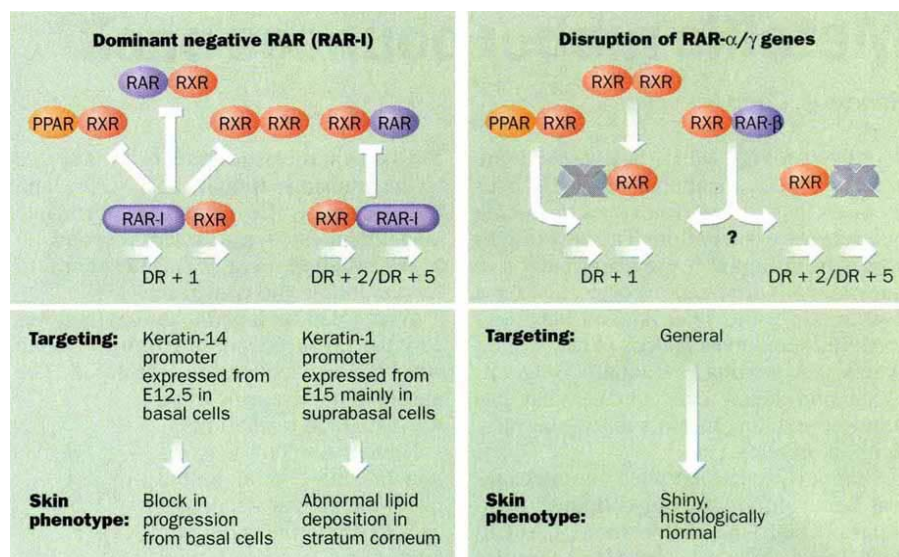
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opment^{8,9}. As RAR- β is absent from epidermis¹², it is surprising that RAR- α /RAR- γ double-knockout mice essentially have skin that is histologically normal, although it looks shinier than that of normal newborns (P. Chambon, personal communication). Disruption of the RXR- α gene leads to early embryonic lethality, which precludes full examination of skin development^{10,11}.

Another genetic approach to the delineation of complex signal transduction pathways is to use dominant negative proteins to interfere with different steps in the pathway. For RAR, this has the advantage that a dominant negative receptor can interfere simultaneously with all isoforms. Furthermore, tissue- and stage-specific enhancers are used to target expression to specific cells at a chosen time in development, thereby avoiding early lethality. Both Saitou *et al.*¹ and Imakado *et al.*² have designed dominant negative RARs that dimerize with RXR and bind retinoic acid response elements, which suggests that these receptors act by forming transcriptionally inactive heterodimers that compete for DNA binding with natural RAR-RXR heterodimers^{13,14}. However, these dominant negative receptors are not fully specific for disrupting RAR function and will interfere with binding of RXR homodimers and peroxisome proliferator-activated receptor (PPAR)-RXR heterodimers to DR+1 elements^{2,15}.

Saitou *et al.*¹ used the keratin-14 promoter to overexpress a dominant negative RAR (RAR- α in which a glycine at residue 303 is replaced by glutamic acid; G303E) in the basal cell layer of skin during embryogenesis and found that the effects on skin formation were dramatic. Keratin-5-expressing basal cells now comprise two layers instead of one, while formation of mature suprabasal cells is hindered, leading to a very thin, dry and fragile skin; hair formation is also delayed. These results suggest that one of the roles of RAR in epidermal keratinocytes is to promote commitment of proliferating basal cells to keratin-1/10-expressing post-mitotic cells. As it is unknown whether the RAR- α (G303E) protein persists in the suprabasal cells, it is unclear whether the phenotype is due just to a block at this stage rather than to continuous action of the dominant negative receptor in suprabasal cells.

Imakado *et al.*² express a dominant negative RAR (RAR- α with residues 404–462 deleted) with the keratin-1 promoter that directs expression predominantly to suprabasal cells¹⁶. This resulted in newborn mice with shiny skin and subtle histological changes affecting only the cornified layer because of abnormal lipid deposition. But this abnormality severely affected barrier function and led to death within 24 hours of birth. Collec-



Using genetics to determine retinoid action in epidermis. Left, the dominant negative RAR (RAR-I) binding as a heterodimer with RXR to an element consisting of a direct repeat separated by one base pair (DR+1), or to DR+2 and DR+5 sites. RAR-I selectively blocks RAR-RXR heterodimers on DR+2 and DR+5 sites, and also blocks PPAR-RXR heterodimers and RXR homodimers on DR+1 sites. When overexpressed, RAR-I might also titrate out RXR that is available for interaction with the receptors for thyroid hormone T_3 and vitamin D, and so interfere with these pathways. The figure shows that different targeting results in different phenotypes. Right, disruption of RAR- α and RAR- γ genes is expected to disable RAR signalling on all sites unless RAR- β is upregulated (indicated by a question mark). It is also possible that PPAR-RXR heterodimer and RXR homodimer binding to DR+1 sites is increased owing to absence of RAR-RXR heterodimers.

tively, these results suggest that retinoids (and/or PPAR ligands) may have at least two distinct functions in epidermal differentiation, first during commitment of basal cells to suprabasal cells, and subsequently during formation of stratum corneum.

How does overexpression of RAR- α (G303E) in basal cells of epidermis dramatically suppress epidermal differentiation while mice homozygous for disruption of both the RAR- α and RAR- γ genes have histologically normal skin? First, the possibility of compensation in the RAR- α /RAR- γ double-knockout mice must be considered, as a result of upregulation of RAR- β for example. Alternatively, some of the caveats of the dominant negative approach must be examined. The RAR- α (G303E) protein could have partial agonistic activity, especially on certain DNA-binding sites. Transgenic mice overexpressing wild-type RAR- α have normal skin, therefore arguing against this possibility¹. Likewise, normal skin formation in mice overexpressing a dominant negative receptor for the thyroid hormone T_3 in epidermis¹ undermines the interpretation that RAR- α (G303E) interferes with related signalling molecules such as the receptors for T_3 and vitamin D, which interact with RXR and have been implicated in epidermal function. However, it is possible that overexpressed RAR- α (G303E) behaves unexpectedly in the cell by interacting with DNA sites or proteins not involved in the retinoid pathway.

The results of Saitou *et al.*¹ and Imakado *et al.*² prompt a re-evaluation of the role of retinoids in epidermal development, yet the inherent lack of specificity of dominant negative RARs emphasizes the importance of using complementary approaches to define specifically the effects of retinoid receptors. Examination of the skin phenotype of mice with combinatorial and conditional knockouts of their retinoid receptors is called for to understand fully how retinoids function in epidermal development. □

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Metallic rubber bounces back

Robert W. Cahn

A PHENOMENON which has puzzled the world's metallurgical community for over 60 years has at last found a convincing quantitative explanation. The new insight comes from Japan^{1,2}, the phenomenon in question was first reported in 1932 by a Swede and it has been kicked back and forth between investigators in the United States, Argentina, Russia, Belgium, Spain and Japan ever since. What has required explanation is rubber-like elasticity in an alloy.

Ölander³ in 1932 studied the mechanical behaviour of a gold-cadmium compound close to the composition of AuCd. This compound, after high-temperature homogenization, undergoes a martensitic transformation on cooling below about 60 °C (the martensitic, or M_S , temperature). The cubic high-temperature ('parent') phase converts into a more complex phase by a process of crystallographic shear; because of the high symmetry of the cubic parent phase, any of 24 distinct but crystallographically similar shear planes, or variants, can operate. The process is just the same as that which is responsible for the hardening of red-hot carbon steel when it is quenched into water.

If the cooled alloy is now aged for some hours at ambient temperature and is then stressed in tension, it will stretch quasi-elastically at a modest stress and with an extraordinarily low elastic modulus, apparently like rubber, and quite unlike an ordinary metal, to a strain of several per cent, and most of the strain will disappear when the stress is removed. This is rubber-like elasticity. (Quasi-elasticity would be a better term, as not quite all the strain is recovered). Ölander recognized that the process is due to the growth of one of the 24 martensitic variants at the expense of all the others: the chosen variant is the one whose associated shear strain most effectively responds to the direction of the applied tension.

Thus the quasielastic strain is actually due to the transformational shear strain, and the process is Le Chatelier's Principle made manifest — the system responds to a perturbation in a way that tends to accommodate the constraint.

What Ölander failed to understand was why the favoured variant contracts again when the applied tension is released. The nature of the restoring force has remained mysterious.

Rubber-like elasticity is just one of several linked phenomena that together characterize thermoelastic martensitic transformations, a subset of the large family of shear-mediated transformations. This term was coined in 1949 by the Russians Kurdymov and Khandros in a renowned study⁴: the microstresses generated by localized shears on different variant planes are accommodated by a local pattern of elastic strains, without any local plastic distortion. Such an alloy can transform equally as a result of temperature change or of applied stress; hence the name 'thermoelastic'. Such alloys can also undergo shape-memory behaviour: at a suitable temperature, close to M_S , an applied stress can force the alloy to transform martensitically and thereby to change its shape substantially (because only a subset of variants, or even just one variant, is activated by the stress). Subsequent heating will reverse the transformation and the shape change is reversed.

Such alloys, always with long-range atomic order (nowadays, specially designed copper alloys are preferred) are used for varied thermal control devices in

cars, for leak-tight tube couplings and other applications⁵. The kind of shape-memory effect — there are several varieties — always involves a martensitic phase change and the nature of the restoring force is well understood. The pattern of elastic microstresses resulting from the forward transformation is responsible for driving the reverse transformation, in the sense that the original orientation of the parent phase is precisely restored. This whole complex of phenomena has recently been concisely reviewed by Wayman and Inoue⁶.

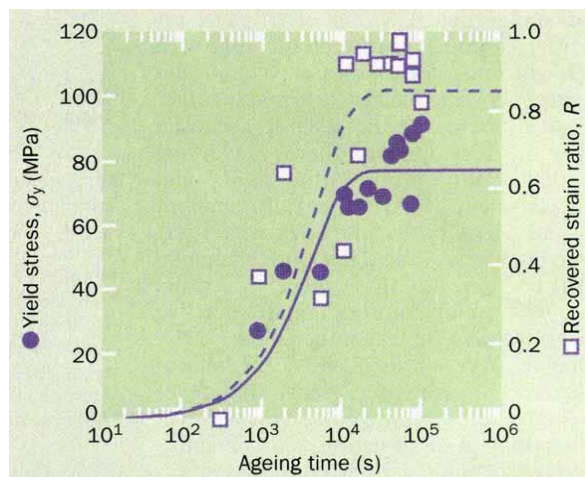


FIG. 2 Apparent yield stress, σ_y , and the ratio of the recovered strain to the imposed strain, R , as a function of ageing time at room temperature for the quenched alloy.

Ölander's alloy in fact underwent shape-memory behaviour if it was stressed immediately after cooling from high temperature; the rubber-like behaviour only appeared after prolonged ageing. However, after ageing, the alloy no longer undergoes any phase transformation when it is stressed, because the martensitic phase has become stabilized: there is only growth of one martensitic variant at the expense of the others as stress is increased. No local microstresses arise during this process, and so they cannot be held responsible for the restoring force.

The new interpretation of rubber-like elasticity began with an experimental study by Tsuchiya and colleagues¹, working at Hokkaido University in Japan. Their alloys were a commercial type of shape-memory alloy, of composition $\text{Cu}_{69}\text{Zn}_{14}\text{Al}_{17}$, and another containing slightly more zinc. Both the parent phase and the martensitic product are fully ordered. The first alloy was prepared in the form of a single crystal, the latter as a polycrystal. As rubber-like elasticity is much more pronounced when a single crystal is used, only the first alloy is discussed here. The crystal was homogenized at 1,143 K and then either quenched into water or slowly cooled. Figure 1 shows how the tensile stress-strain curve of the quenched alloy changes as a

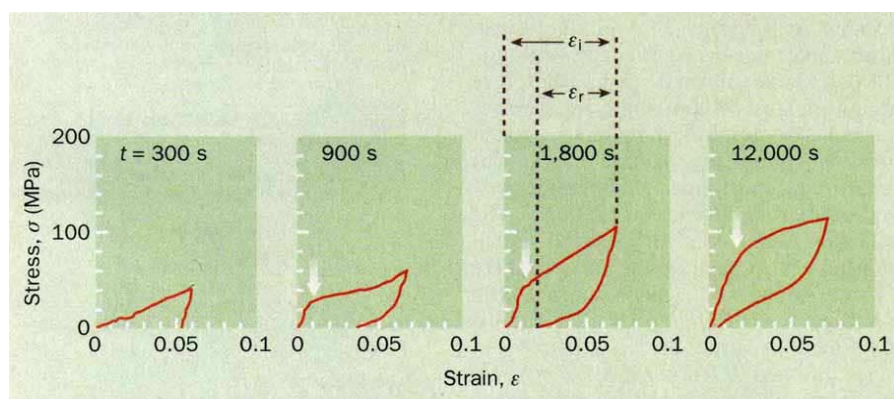


FIG. 1 Tensile stress-strain curves of the alloy used by Tsuchiya *et al.*¹ after various ageing times at room temperature.

function of ageing time at ambient temperature following the quench. Figure 2 shows how the yield stress σ_y (denoted by arrows in Fig. 1) increases with ageing time; this graph also shows that the ratio, R , of recovered strain ϵ_r to imposed strain ϵ_i is affected by ageing. When the same alloy is slow-cooled from high temperature instead of being quenched, both the hardening and the recovery of strain during the stress cycle develop much more sluggishly: very long room-temperature ageing is needed (more than 10^5 seconds).

Separate experiments showed clearly that the rubber-like behaviour, associated with a high σ_y and a high R , are indeed linked to the stabilization of the martensitic phase against reversion to the high-temperature phase.

The second paper from Hokkaido, by Marukawa and Tsuchiya², sets about interpreting these findings. They attribute the rubber-like elastic behaviour to a state of short-range (atomic) order (SRO), superimposed on the less-than-perfect long-range order (LRO) in the alloy. The LRO is imperfect because the high-temperature phase is annealed at a high temperature and therefore is not completely ordered, and its state of imperfect LRO is directly inherited by the martensitic daughter phase. The quenched alloy (but not the slowly cooled alloy) is rich in excess, quenched-in vacancies, sufficient to permit diffusive rearrangement of nearest-neighbour atom pairs.

What happens is that some 'wrong' nearest-neighbour bonds, inherited via the martensitic transformation on cooling, are 'corrected' during room-temperature ageing. Because of the crystallographic shear that executes the phase transformation, the 12 nearest neighbours of any one atom in the parent phase become divided into some nearest and some second-nearest neighbours in the daughter phase, and this division is different for each of the 24 shear variants. If now one variant grows at the expense of another, during tensile loading, a proportion of erstwhile nearest neighbours is instantly converted into second-nearest neighbours, and vice versa. The *equilibrium* proportion of unlike neighbours is different for nearest and second-nearest neighbours, and thus the result of the changeover of neighbours is to increase the overall bond energy in the second variant. This creates an effective driving force (equivalent to an energy gain per mole transformed), when the tensile stress is released, for the second variant to be reabsorbed by the first; in other words, the pseudoelastic forward strain is reversed when the stress is released.

Marukawa and Tsuchiya² were able to estimate this 'driving force' and give an upper limit of 157 J per mole transformed. From the earlier experiments, they estimate an actual 'force' of about 50 J per mole. This must be somewhat less than the

calculated quantity, as the extent of ageing is limited by the supply of quenched-in vacancies. Given the unavoidable uncertainties in the calculation, this represents satisfactory agreement and a long-standing mystery may be regarded as solved.

The detailed measurements and theory presented by the Japanese researchers develop and refine an earlier version of the SRO theory, advanced by an Argentine group under M. Ahlers some years ago⁷. The Japanese experiments are more detailed, but the Argentine group has a claim to be regarded as the true initiator of the concept.

The experiments might with advantage be taken one step further. It has been firmly established from magnetic studies⁸ and more recently by precision X-ray diffraction⁹ that directional (anisotropic) SRO, with one preferred axis for unlike nearest neighbours, can be induced in an imperfectly ordered intermetallic compound or in a metallic glass by annealing in a magnetic field or under an elastic stress. If, therefore, the alloy is aged at ambient temperature as before, but after about 10^4 seconds (when the yield stress has in-

creased enough) a stress of, say, 50 MPa is applied and ageing is continued under stress, then the rubber-like elasticity could well be further increased as a result of the induced directional SRO. Such a test would be well worth performing, and might help establish the SRO concept beyond cavil. □

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TRANSCRIPTIONAL REGULATION

Cyclins in initiation

Elizabeth M. O'Neill and Erin K. O'Shea

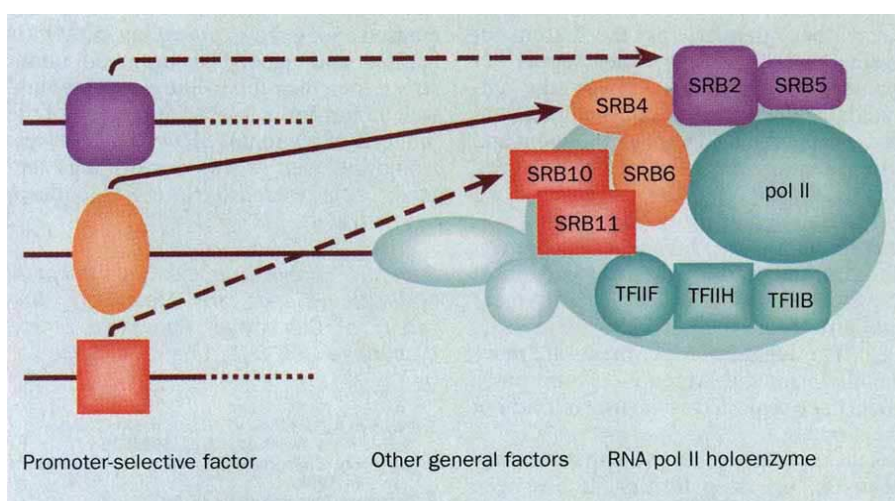
CYCLINS and their catalytic partners, the cyclin-dependent kinases (CDKs) have long been known as key regulators of the cell-division cycle and are now recognized as important participants in other cellular processes. For example, a CDK/cyclin complex functions in a signal transduction pathway that senses phosphate availability in yeast, and another such complex is a component of the transcription factor TFIIF. Now the pair emerge in a new guise as subunits of the yeast RNA polymerase II holoenzyme: dubbed SRB10 and SRB11 by Liao *et al.*, their effects on transcription are described on page 193 of this issue¹.

The carboxy-terminal domain (CTD) of the largest subunit of RNA polymerase II (pol II) is phylogenetically highly conserved. Deletion mutations that remove the CTD are lethal to cells, and partial truncations give rise to a cold-sensitive phenotype^{2,3}. SRB10 and SRB11 are members of a group of genes which suppress this phenotype when they are mutated. Two other groups have identified alleles of SRB10 and/or SRB11 during searches for genes involved in two different cases of regulated repression of transcription: Wahi and Johnson⁴ show that SRB10 (alias ARE1) helps to repress transcription of the α -specific genes in α -cells; Kuchin *et al.*⁵ have isolated alleles

of SRB10 (SSN3) and SRB11 (SSN8) that are involved in repression of genes in response to glucose. Two additional proteins that are required for transcriptional repression of these and other genes in yeast, SSN6 and TUP1, are recruited to promoters by interactions with specific DNA-binding proteins⁶.

A picture is emerging in which SSN6/TUP1-dependent repression may be mediated by some common mechanism requiring SRB10 and SRB11. But results from two of these groups^{1,5} also implicate SRB10 and SRB11 in transcriptional activation (of the *GALI* promoter). Interpretation of these *in vivo* results is complicated by the fact that the repression effect could be an indirect result of an activation defect or vice versa. *In vitro* reconstitution experiments will be required to determine whether SRB10 and SRB11 have a direct role in activation and/or repression.

How does SRB10/SRB11 function to modulate transcriptional activity? In early studies in which pol II transcriptional activation was reconstituted *in vitro*, the proteins required for initiation of messenger RNA synthesis were neatly divided into two categories. One class, the general factors, are needed at all promoters for 'basal' transcription, and the second class, the promoter-selective factors, act through specific DNA sequences located



A model depicting possible interactions between specific SRB proteins and different promoter-selective factors leading to alterations in gene expression. In the case of SRB10 and SRB11, interactions may lead to repression and/or activation, depending on the nature of the promoter-selective factor (see text for details). Removal of any one of the SRBs would lead to loss of tightly regulated gene expression only at the genes regulated by that particular subset of promoter-selective factors.

in the promoters of genes. It is thought that the promoter-selective factors communicate with the general factors to stimulate 'activated' transcription. Promoter-selective repressors are likewise thought to communicate directly with the general factors.

Another class of transcription factor has recently been identified⁷. These factors, termed mediators or coactivators, are dispensable for basal transcription and incapable of recognizing specific DNA sequences. They form stable complexes with certain of the general factors, and are necessary for mediating activation and perhaps repression through direct contact with promoter-selective factors. Furthermore, certain coactivators appear to be specific for a subset of promoter-selective factors.

The yeast pol II holoenzyme is a large, stable complex composed of RNA polymerase II, a subset of the general transcription factors, including TFIIB, TFIIF, TFIH and a group of other proteins, many of which have turned out to be identical to the SRBs^{3,8}, and which function as mediators⁹. Thus, it seems that the role of the SRBs is to enhance the ability of the general factors to respond to the promoter-selective factors, whether they are activators or repressors.

Genetic data regarding the SRBs support this model, and suggest distinct roles for certain subsets of the SRBs. As some alleles of the SRBs identified originally were dominant and others were recessive, deletion mutants of the *SRB* genes can have different phenotypes¹⁻³. Strains deleted for *SRB10* or *SRB11* are viable and exhibit a set of phenotypes common to strains defective in *SSN6/TUP1*-mediated transcriptional repression^{4,5}, suggesting that they are important in repression. A second class of SRB deletions, repre-

sented by *SRB2* and *SRB5*, have a set of phenotypes similar to that of the partial CTD truncation used in the *SRB* mutant hunt. *In vitro* and *in vivo* data show that these two SRBs are important for mediating activation^{2,3}. A third class of SRBs, represented by *SRB4* and *SRB6*, is required for viability³. Perhaps these different phenotypes *in vivo* reflect defects in the response to different subsets of promoter-selective factors, as has been observed *in vitro* for metazoan coactivators. This in turn would result in defects in the expression of distinct subsets of genes leading to different pleiotropic phenotypes.

To test the model in which these three classes of SRBs mediate the action of distinct subsets of promoter-selective factors (see figure) will require more work, including an investigation of the SRB dependence of *SSN6/TUP1*-mediated repression *in vitro*. But the powerful combination of genetic and biochemical studies feasible only in yeast will lead to a more detailed understanding of the mechanism of regulation of eukaryotic gene expression. □

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DAEDALUS

Protein portfolio

PROTEINS are renowned for catalysing chemistry, but they are good at physics as well. Thus the bacterial protein 'ice-plus' often gets into strawberries, and triggers their freezing in even a mild frost. The arctic ice-fish synthesizes a converse protein, which inhibits the freezing of its tissues even in the coldest conditions. Daedalus is now feeding ice-fish on strawberries, and spraying strawberries with ice-fish juice, to discover which of these two powerful proteins trumps the other. The result should either be a biological frost-protection system for plants, or a new technique of arctic fishing.

The principle can clearly be extended. Only a few proteins nucleate or inhibit the crystallizing of water; but there are millions of other proteins, and millions of other possible liquids — organic solvents, chemicals which melt at modest temperatures, or supersaturated solutions which need a specific nucleating agent to start them crystallizing. Almost every protein should nucleate some liquid or other, and conversely there should be some protein which nucleates any given liquid. DREADCO chemists are now exploring a vast matrix of liquids and proteins to look for such matches. With luck the guiding principles will soon emerge, allowing the team to match liquids to nucleating proteins at a great rate.

The result will be a splendid new hypersensitive protein-identification system, to supplement or even replace electrophoresis. A mere nanogram or so of material can set off nucleation. To study the distribution of some protein in a biological sample, just spray it with the correct supercooled liquid and note where the crystallization starts.

But Daedalus wants to apply the idea to human identification. Each of us has our own, genetically controlled, personal portfolio of proteins. By waving a finger over a set of liquids in a well-plate, shedding a few dead cells into each, you could prove your identity from the ones which crystallize. Heat the plate again to remelt the crystals and denature the proteins, and it would be ready for the next customer. This quick, cheap, simple system could distinguish over a million people on a mere twenty well-chosen liquids; thirty-one could identify everyone on the planet. Nobody could alter his biochemistry to impersonate yours: the system would be quite secure. It could replace passports, ID badges, plastic cards, signatures, pin-numbers and all the other identifiers now cumbering your life. Should you come to a bad end, it would even identify your corpse.

David Jones

Ferromagnetism and EMFs

SIR — The question of whether weak, extremely low-frequency electromagnetic fields (EMFs) can cause cancer always generates heated debate (see, for example, refs 1–3). In addition to epidemiological studies, a substantial body of literature exists on EMF stimulation of cells grown *in vitro* (for example, refs 4, 5). Although numerous effects have been reported, many have been difficult to replicate (see refs 6, 7), and no clear biophysical mechanism has emerged. Many of the proposed mechanisms, like ion cyclotron resonance⁸, have drawn criticism for being physically unrealistic (see ref. 9).

From developments in a totally unrelated field, there may be a much simpler, as yet overlooked, mechanism for explaining many of these *in vitro* EMF cellular effects. For the past two decades, the study of the biologically precipitated ferrimagnetic mineral magnetite (Fe_3O_4) has relied heavily on the use of ultrasensitive superconducting quantum interference device (SQUID) magnetometers to quantify trace levels of magnetite in various biological and laboratory materials^{10,11}. It rapidly became clear that unique clean-laboratory techniques were required for this work because of the ubiquitous presence of ferromagnetic contamination. This contamination included ferromagnetic particulates present not only in the dust in the air, but also adsorbed onto the surfaces of laboratory equipment, present within glass and plastics, and even in reagent-grade laboratory chemicals and water.

We have encountered the same problem in our recent attempts to grow cells in tissue culture for an investigation of their magnetic properties. It is customary to use disposable, pre-sterilized plastic labware (flasks, pipettes, centrifuge tubes, and so on) and commercially prepared culture media in tissue-culture experiments because of their convenience and the assumption of a high level of quality control and cleanliness. We have found that none of these materials is free of ferromagnetic particulate contamination. Liquid-transfer manipulations, typical of cell-culture protocols, wash these particles from the surfaces of flasks and pipettes,

and concentrate them with the cells during centrifugation. As an example, in a sham experiment we used 50 ml of leukocyte culture medium to rinse ten plastic T-250 flasks, ten 10-ml pipettes and ten 50-ml centrifuge tubes. After final centrifugation, we detected the equivalent of 160 ng magnetite in the rinsate, and the magnetic data indicated that the contaminants are small particles, usually in the sub-100-nm size range. As 160 ng magnetite equates to about 32 million 100-nm³, this can be compared to the approximately 1 million cells that would have been produced in an equivalent culture volume.

Magnetite particles, 100 nm in diameter, either naked or coated with bovine serum albumin, are readily taken up by human white blood cells, including non-phagocytic lymphocytes as well as phagocytes¹². Because the ferromagnetic particles interact strongly with magnetic fields, their presence in cell cultures, at a number density far higher than that of the cells, may provide a simple mechanism to account for links between EMF exposure and *in vitro* biological effects. A simple calculation shows that the mechanical energy present in a single 100-nm magnetite crystal exposed to a 60-Hz, 0.1-mT magnetic field is many times the thermal background noise¹³. Such particles, if adsorbed on cell surfaces or ingested by the cells, could conceivably transfer this energy to contiguous cell structures such as mechanically activated ion channels (which operate with a gating force close to the thermal noise limit^{14,15}), and thereby alter cytoplasmic ion concentrations sufficiently to produce the observed biological effects.

We are not aware that the authors of any of the published studies of *in vitro* EMF effects have either controlled for, or attempted to reduce the levels of, ferromagnetic contamination. Although this is understandable, because the particles are difficult to detect and quantify except by sensitive magnetometry, their existence should not be ignored. *In vitro* studies may ultimately provide the information that will explain the connection between EMF exposure and biological effects, and as

such they constitute roughly half of the projects at present being sponsored by the 5-year, \$65 million NIEHS/DOE research programme on the biological effects of EMF. However, any effect of EMF exposure on cultured cells, if it is due to the presence of ferromagnetic contaminants, would have no relevance to *in vivo* biology. Data used to establish human exposure standards to electromagnetic fields must rely on properly controlled experiments.

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Extant fauna of ancient carbon

SIR — We have found that organisms of the rich, freshwater biological community involving sponges, flatworms and other benthic species near a thermal vent at the bottom of Lake Baikal are built of ancient carbon lacking ¹⁴C.

The vent occurs at a depth of 420 m in Frolikha Bay (55°31' N, 109°46' E)^{1,2}. It was found earlier that the carbon of its benthic organisms was produced by methanogenic bacteria, as revealed by the very small values of $\delta^{13}\text{C}$ (–60 to –72‰)². However, the age of this carbon was not known.

Using a Tandemtron accelerator mass-spectrometer, we measured the contents of ¹⁴C in carbon of two *Bdellocephala* flatworms and a sponge collected on a bacterial mat of Frolikha vent, and found them to be equal to 0.43, 0.34 and 0.28 of that typical of modern organic matter, corresponding to apparent radiocarbon ages of $6,860 \pm 260$, $8,740 \pm 80$, and $10,200 \pm 220$ years before present (BP), respectively.

Hence, about 60–70 % of the carbon of the near-vent organisms has originated from ancient methane, rather than from modern atmospheric CO₂ due to photosynthesis or methanogenesis. The source of ancient carbon was also not limestone, as is sometimes the case in freshwater systems, since the uppermost layer of Baikal sediments is known to have an apparent radiocarbon age of less than 1,000 years BP at many locations (see ref. 3 and T. N. *et al.*, unpublished data).

Frolikha vent arises from meteoric water seeping through Baikal sediments⁴ that are known to contain high concentra-

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tions of methane; gas hydrates are ubiquitous under the floor of the lake, as shown by seismic profiling⁵. The small dependence of the near-vent community on photosynthesis suggests that vents of this kind could have been important in the nascence of the unique faunistic complex of Lake Baikal consisting of 1,500 endemic species: vents could have many times served as refuges under unfavourable climates, and sources of species radiation under more favourable ones during the 20-million-year-long history of the lake. Communities of organisms built of ancient carbon are not uncommon in a marine ecosystem⁶, but this is the first time they have been found in a freshwater ecosystem.

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Another obese gene function

SIR — The cloning of the *obese* (*ob*) gene¹ is indeed a breakthrough in obesity research. As Rink highlighted in News and Views², the evidence strongly suggests that the normal *ob* protein is a previously undescribed hormone which regulates satiety. I would like to comment on another potential function of the *ob* gene.

When allowed free access to food, *ob/ob* mice eat more than normal *ob/ob* mice and develop obesity. However, the lean body mass of *ob/ob* mice is lower than *ob/ob* mice, and shows characteristics of stunted animals³. Furthermore, food restriction to the level of the normal *ob/ob* mouse does not reduce adiposity of the *ob/ob* mouse,

but it only causes a further decrease in lean body mass with little change in fat/lean body ratio compared with the *ob/ob* mouse fed without food restriction⁴. The same results are observed in the *falfa* rat, another genetic model of obesity caused by a single recessive gene⁵. The *falfa* rat is thought to have a homologous defect to the *db/db* mouse⁶, which may have a defective receptor for the *ob* protein².

These effects of the *ob* gene cannot be explained by suppression of appetite and subsequent reduction of food intake. Rather, the observations indicate that the *ob* protein regulates the energy partition between fat deposits and the lean body by some mechanism(s) not secondary to the effect on food intake, and that a defect in *ob* gene function causes an increased energy flow toward fat accumulation even at the expense of lean body growth.

In humans, uncommon obesity caused by a single gene has been reported. On the other hand, humans generally show increased lean body mass when the degree of obesity increases; and the treatment of obesity by energy restriction effectively reduces body fat without reducing lean body mass. Thus, it will be particularly interesting to determine the role of the human homologue of the mouse *ob* gene in human obesity. The discovery of the *ob* gene sequence will soon lead to the answer to this question. Even if this gene has little relevance to most obesity in humans, the potential use of the *ob* protein for the treatment of obesity will remain.

Manabu T. Nakamura

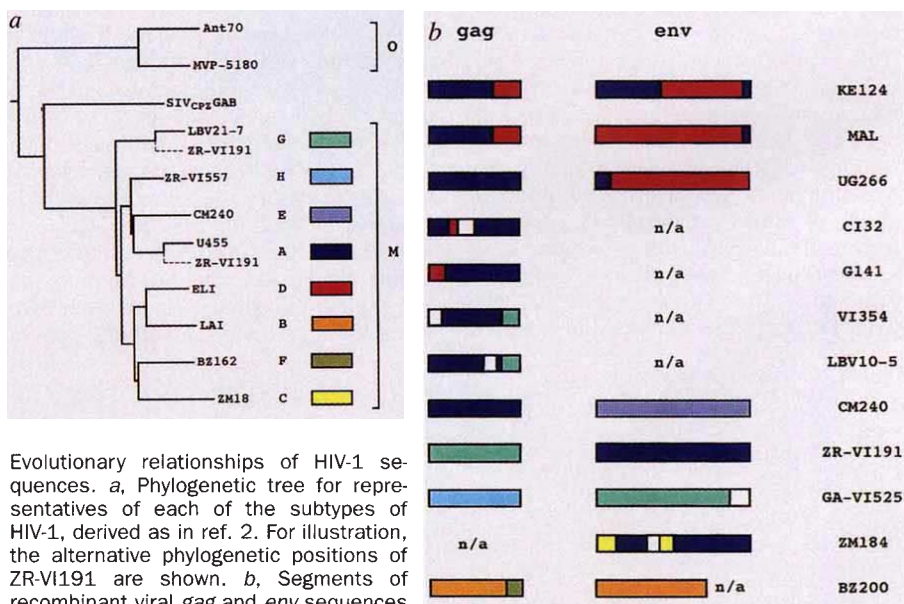
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Recombination in HIV-1

SIR — Globally circulating strains of human immunodeficiency virus type 1 (HIV-1) exhibit extreme genetic diversity^{1–5}. Phylogenetic analyses (*a* in the figure) have revealed two distinct 'groups' (M and O), and numerous 'sequence subtypes' within the major group M. Even though retroviruses (such as HIV-1) are highly recombinogenic⁶, recombination among viruses from different subtypes has not been considered to be a significant source of new variation in HIV-1 because evidence for coinfection with multiple divergent HIV-1 strains has remained rare⁷. Here we report an extensive analysis of published HIV-1 sequences which reveals a surprisingly large number of apparently recombinant viruses. This find-

ing has immediate consequences for our understanding of HIV-1 pathogenesis and for vaccine development⁸, and of course implies that coinfection with divergent HIV-1 strains is not as rare as previously thought.

Recombination can be detected when different genes, or different regions within the same gene, are placed by phylogenetic analysis into different sequence subtypes. A single HIV-1 isolate (MAL) has long been suspected to be recombinant, and we have recently described⁹ a detailed analysis of this viral genome in which the crossover points were localized. Here we have applied the same techniques to examine all HIV-1 isolates for which near-full-length *gag* or *env* sequences have been



Evolutionary relationships of HIV-1 sequences. *a*, Phylogenetic tree for representatives of each of the subtypes of HIV-1, derived as in ref. 2. For illustration, the alternative phylogenetic positions of ZR-VI191 are shown. *b*, Segments of recombinant viral *gag* and *env* sequences belonging to different subtypes; colour coding is as in *a*. Some segments (in white) cannot be designated as belonging to any currently known subtype. Localization of the breakpoints between regions of differing phylogenetic affinity is described in the table.

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deposited in the database. Phylogenetic trees were constructed using various regions of the *gag* and *env* sequences: putative recombinants were identified as those sequences falling in significantly discordant positions (as assessed by bootstrapping) in different trees, and these sequences were subjected to further investigation.

Breakpoints between genomic regions with different phylogenetic histories were localized by a method adapted from ref. 10. Among 114 viruses analysed, at least 10 appear to be recombinants of sequences from different group M subtypes (*b* in the figure). In many cases recombination breakpoints were found within genes (see table), and the recombination events appear to have involved multiple crossovers, as seen (under laboratory conditions) for other retroviruses⁶. For most of these examples, the probabilities of observing (by chance) these discordant phylogenetic positions for different genes (summarized in *b* of the figure), or the nonrandom distribution within genes of sites supporting alternative phylogenetic positions (see table), are very low. However, for completeness, we have also included two examples which are not, as yet, definitive. LBV10-5 falls as an outlier to subtype A if the entire *gag* sequence is analysed, in a position similar to other viruses (for example KE124) with mosaic *gag* genes. The 3' end of the LBV10-5 sequence appears to be subtype G, but the test yields $0.05 < P < 0.10$; this may be because this 3' region is fairly short, and sequencing the region downstream of *gag* could resolve this. CM240, as well as a number of other closely related viruses from Thailand, have been placed³ in *gag* subtype A, but classified separately⁴ as (the sole representatives of) *env* subtype E, for which no *gag* equivalent is yet known. CM240 is probably recombinant as it is relatively much more distant from subtype A viruses in *env* than in *gag*, but it is conceivable that this could result (without recombination) from different evolutionary rates in different regions of the genome.

Thus all eight subtypes of HIV-1 group M so far described appear to have been involved in recombination events, but no sequences were found that were hybrids of group M and group O viruses. All of

Isolate	Origin	Gene	Subtype	Region	Informative sites			P
					1	2	SIV _{CPZ}	
KE124	Kenya	<i>gag</i>	A	1-1050	35	3	4	} 0.000
		<i>gag</i>	D	1083-1477	0	14	3	
		<i>env</i>	A	1-1065	30	4	4	} 0.000
		<i>env</i>	D	1096-2435	4	46	10	
		<i>env</i>	A	2480-2580	7	1	1	} 0.000
MAL	Zaire	<i>gag</i>	A	1-1068	34	6	4	} 0.000
		<i>gag</i>	D	1101-1518	4	12	1	
		<i>env</i>	D	1-2435	9	61	30	} 0.005
		<i>env</i>	A	2482-2580	5	1	0	
UG266	Uganda	<i>env</i>	A	1-199	10	1	4	} 0.000
		<i>env</i>	D	252-2556	9	62	11	
CI32	Cote d'Ivoire	<i>gag</i>	A	1-333	10	4	3	} 0.081
		<i>gag</i>	D	417-423	0	4	0	
		<i>gag</i>	A	456-1477	21	7	10	} 0.037
G141	Gabon	<i>gag</i>	D	1-261	1	13	5	} 0.000
		<i>gag</i>	A	306-1459	38	7	5	
VI354	Gabon	<i>gag</i>	A	1-1154	38	9	14	} 0.020
		<i>gag</i>	G	1245-1465	1	5	1	
LBV10-5	Gabon	<i>gag</i>	A	1-1166	34	7	12	} 0.087
		<i>gag</i>	G	1209-1483	4	6	3	
ZM184	Zambia	<i>env</i>	C	1-328	0	11	0	} 0.000
		<i>env</i>	A	363-1053	13	2	12	
		<i>env</i>	C	1068-1263	1	8	1	} 0.003
		<i>env</i>	A	1270-2547	33	7	13	
BZ200	Brazil	<i>gag</i>	B	1-1227	38	1	6	} 0.000
		<i>gag</i>	F	1253-1474	1	11	2	

Localization of intragenic crossover breakpoints between regions belonging to different subtypes in recombinant HIV-1 DNA sequences. For each sequence, phylogenetic analyses were performed using four taxa: the putative recombinant; two consensus sequences, each derived from nonrecombinant members of the subtypes seemingly involved in the recombination event; and an outgroup (SIV_{CPZ}GAB; *a* in the figure). The number of phylogenetically informative sites supporting the grouping of the recombinant sequence with each of the other three sequences are given. The regions were chosen so as to maximize the statistical significance (assessed by a heterogeneity χ^2 with 1° of freedom) of the difference in the distribution of sites supporting phylogenies 1 and 2: probability values (assessed from simulations) pertain to the comparisons bracketed. Regions of unclear ancestry within the *gag* genes of CI32 (at 456-852), VI354 (1-214) and LBV10-5 (762-1062), and the *env* gene of ZM184 (798-1021) (*b* in the figure) have been subsumed within neighbouring regions, leading to a probable underestimation of significance values. All sequences were obtained from the GenBank/EMBL/DBJ database.

the putative recombinants originated from geographic regions where multiple sequence subtypes are known to co-circulate, including central Africa, South America and southeast Asia³⁻⁵. Whether the frequencies of the various mosaic genomes are representative of the prevalence of recombinants at large awaits more systematic study. Future analyses should also aim to verify the presence of mosaic genomes *in vivo*, to exclude the possibility of tissue culture and/or PCR artefacts (all sequences available for this study were derived from cultured viruses).

The surprisingly high frequency of mosaic HIV-1 sequences in the database implies that a substantial proportion of individuals can become coinfecting with HIV-1 strains belonging to different sequence subtypes, and that recombination between these genomes can occur *in vivo* to generate biologically active viruses, often with hybrid *gag* or *env* proteins. These results raise pressing questions con-

cerning the global frequency of such recombinants, their biological significance, and their impact on vaccine design and evaluation. They prompt investigation of the circumstances under which coinfection (either by simultaneous transmission of divergent viruses, or by successive superinfection) can occur. They also point to the need for natural history studies addressing whether these hybrid HIV-1 genomes have new and significantly altered biological properties. In the con-

Scientific Correspondence

Scientific Correspondence is intended to provide a forum in which readers may raise points of a scientific character. They need not arise out of anything published in *Nature*. In any case, priority will be given to letters of fewer than 500 words and five references.

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text of ongoing efforts at vaccine development, in which vaccine preparations may need to be aimed at individual sequence subtypes⁸, it is clear that diversity surveys must include measures to identify mosaic viruses. Finally, it is important to realize that our present survey almost certainly underestimates the frequency of co- or superinfections, since recombinants of divergent viruses from the same sequence subtype are much more difficult to detect.

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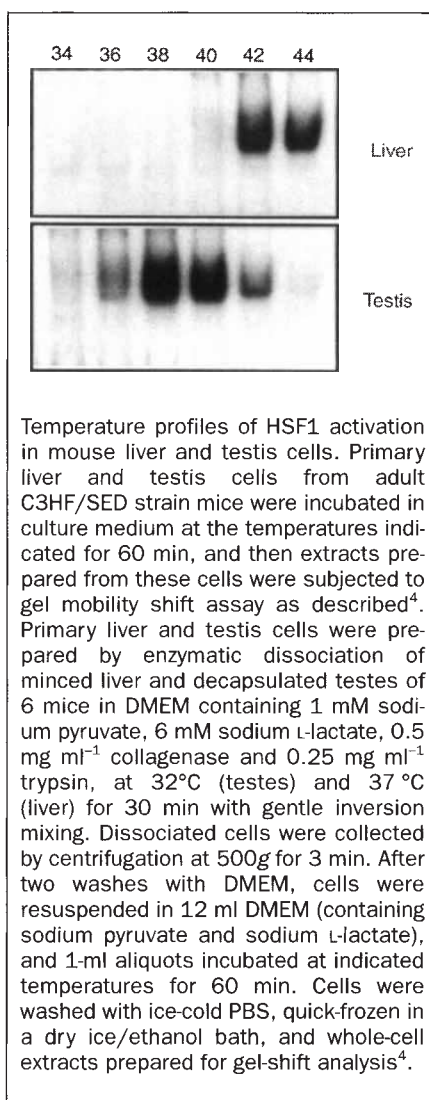
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Altered stress response in testis

SIR — Heat-shock factor (HSF), a transcriptional regulator protein exhibiting heat-activatable DNA binding, mediates the stress-induced expression of eukaryotic heat-shock protein genes (for review see ref. 1). The temperature set-point for HSF activation varies between species, but it is unknown whether this set-point is identical in all cells of a single organism. A unique feature of male gonads of many species is their location outside the main body cavity, so that testis cells have a significantly lower growth temperature relative to cells of other tissues². In the mouse, testis temperature is tightly regulated at 30 °C, 7 °C lower than the body cavity temperature at which tissues such as liver are maintained. Therefore, we sought to determine whether the temperature profile of HSF activation in mouse testis cells is identical to that in liver cells, or whether it is altered in a way that is consistent with the lower growth temperature of this cell type.

Gel-shift analysis demonstrates that HSF DNA binding in liver cells is induced by treatment at temperatures of 42 °C and above (see figure), a temperature profile similar to that observed for mammalian cell lines³. In contrast, testis cells induce HSF DNA-binding activity at significantly lower temperatures, exhibiting low levels at 36 °C, high levels at 38 and 40 °C, and diminishing levels at 42 and 44 °C. Gel-



Temperature profiles of HSF1 activation in mouse liver and testis cells. Primary liver and testis cells from adult C3HF/SED strain mice were incubated in culture medium at the temperatures indicated for 60 min, and then extracts prepared from these cells were subjected to gel mobility shift assay as described⁴. Primary liver and testis cells were prepared by enzymatic dissociation of minced liver and decapsulated testes of 6 mice in DMEM containing 1 mM sodium pyruvate, 6 mM sodium L-lactate, 0.5 mg ml⁻¹ collagenase and 0.25 mg ml⁻¹ trypsin, at 32 °C (testes) and 37 °C (liver) for 30 min with gentle inversion mixing. Dissociated cells were collected by centrifugation at 500g for 3 min. After two washes with DMEM, cells were resuspended in 12 ml DMEM (containing sodium pyruvate and sodium L-lactate), and 1-ml aliquots incubated at indicated temperatures for 60 min. Cells were washed with ice-cold PBS, quick-frozen in a dry ice/ethanol bath, and whole-cell extracts prepared for gel-shift analysis⁴.

shift analysis in conjunction with specific polyclonal antibodies demonstrates that the HSF DNA-binding activity induced in testis cells by incubation at 38 °C, like that found in 42 °C-treated mouse and human cell lines^{4,5}, is composed of HSF1 (data not shown).

These results show that mouse testis cells activate HSF1 at a significantly lower temperature than liver cells, demonstrating that the temperature setpoint for HSF activation does not have a fixed value in a given species, and can vary in a cell-type-dependent manner. These findings are consistent with previous studies suggesting that HSF is not activated in response to absolute temperature experienced by the cell, but rather in response to a change in temperature^{6,7}. In fact, our results suggest a tight coupling between cellular growth temperature and HSF activation temperature. In spite of their 7 °C difference in growth temperatures, HSF activation in both mouse and liver cells occurs at temperatures 4–5 °C above their respective growth temperatures.

What difference exists between cells

which could modulate the HSF set-point? Heat-induced protein denaturation has been proposed to be the signal that triggers HSF activation⁸. Therefore, one explanation is that HSF activation temperature is directly tied to the protein thermal denaturation profile characteristic of each cell, particularly the earliest-occurring thermal transitions⁹. We propose that the lowered HSF activation temperature in testis cells may be related to a reduced thermal stability of one or more proteins expressed in these cells. Likely candidates are testis-specific proteins, whose thermal denaturation profiles could have been shifted during evolutionary adaptation of testis cells to their lower growth temperature.

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Chicken and egg

SIR — I was surprised to read in your 125th anniversary issue¹ the statement “The microwave background radiation, which fills even the corners of the Universe, would psychologically have been more compelling evidence for the Big Bang if it had been predicted before its discovery in 1965.”

Indeed, microwave background radiation was predicted before its discovery, so much earlier that it must have escaped the notice of the editor and many other scientists as well. In fact, as far as I know, the first calculation of the background radiation temperature reported appeared in a brief *Nature* article². The magnitude of the temperature reported at that time was ~5 K. The details of the calculations were published a short time later in several places.

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Social irresponsibility

Daniel S. Greenberg

Molecular Politics: Developing American and British Regulatory Policy for Genetic Engineering, 1972–1982. By Susan Wright. University of Chicago Press: 1994. Pp. 591. \$75, £59.95 (hbk); \$29.95, £23.95 (pbk).

ANYONE who hangs around the science establishment soon finds that its senior ranks teem with characters determined to do things their way, in the name of good science and public benefit, of course; that ostensibly balanced committees are often pre-loaded with like-minded partisans harmonizing over stacked agendas; that the quest for glory, wealth and corporate power does influence science policy; and, finally, that the political process accords science a generously long leash.

Science is not alone in these respects. But the rhetoric of science resounds so loudly with claims of ethical purity, objectivity and social responsibility that the contrast with reality can be jarring. Nonetheless, the dichotomy attracts little public attention, because the virtuosos of committee tactics are generally concerned with the internal affairs of the community.

Occasionally, however, issues spill beyond the committee room and onto the public stage. Susan Wright, a political scientist and historian of science at the University of Michigan, explores the most dramatic of these issues in postwar relationships between science and government: science's self-initiated regulation and subsequent deregulation of recombinant DNA research. This on-and-off process began in 1974 when researchers themselves voiced concern about the risks of runaway microbes in the new world of gene splicing. It was essentially over by 1980, when the all-clear was sounded by virtually all the original alarmists. Many then scolded themselves publicly for what, in their hindsight, came to be regarded as a needless and perilous precedent in restraints on scientific inquiry.

Wright's observations and conclusions about this sequence of events are scathing, and are bound to infuriate the scientific establishment. Her barbs will be especially felt by many present-day researchers and administrators who played important roles in what is generally depicted as an altruistic, if misguided, episode of collective restraint by a research community.

It was far from that, contends Wright, whose thesis runs as follows. Under false colours of social responsibility, scientists eager to get on with recombinant DNA research captured the forum for debate. They then cunningly pursued their own selfish agenda, in disregard of indisputable uncertainties about possible hazards to public safety. Why did they go

through this convoluted and grating process? Because, says Wright, they initially did harbour serious worries about the hazards of their research. But they were motivated, too, she writes, by fear of further inflaming the antiscience mood of the Vietnam period and the rapidly growing environmental movement. The object,

who were to be regulated. A few outsiders were granted membership of RAC. But the motive, Wright says, was "frankly political and self-protective", as reflected in the written comment of a member, Jane Setlow, concerning one such appointment: "I'm not sure this person could contribute to the deliberations, but I am sure that we need one for the purpose of being able to say we have one when there are complaints".

As research proceeded under RAC's advice for containment and other restraints, the research community rang with complaints that the rules were needlessly onerous. Industry challenged the rules as a drag on the economy. A series of meetings concluded that the original

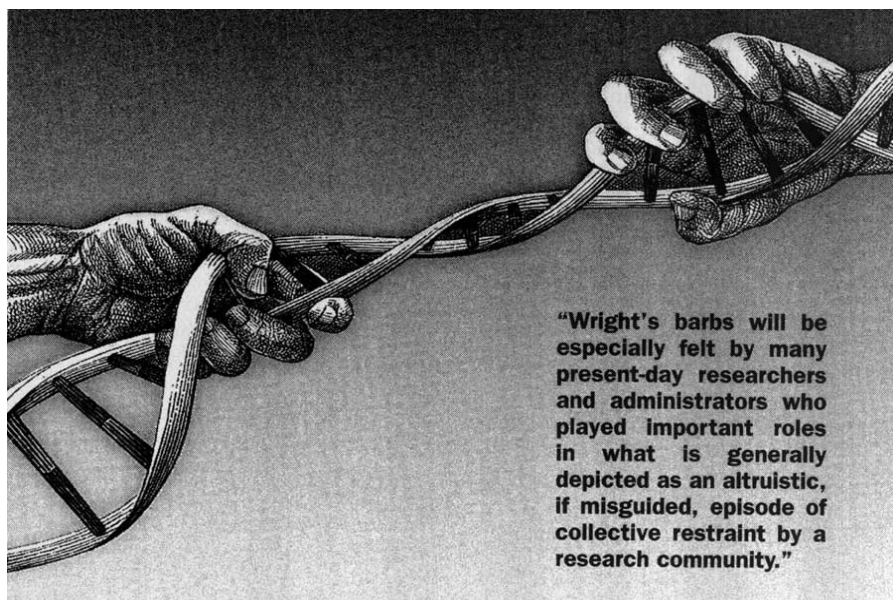


Illustration by Jaye Schlesinger, from the cover of *Molecular Politics*.

she says, was to fend off externally imposed controls by grabbing the issue and adopting a false pose of strict, self-imposed social responsibility.

The landmark event in this saga, the 1975 Asilomar conference, conveyed the impression "of an international community of scientists idealistically moving to restrict their own research and to anticipate its hazards", Wright says. But in reality "the proceedings were also designed to enable research to move forward. . . and this goal was anticipated by the organizers from the beginning". The meeting concentrated on proceeding with research, with scant attention to "broader social, ethical, and policy issues", she asserts.

The task of developing safety regulations was quickly assumed by the US National Institutes of Health (NIH), the main source of funds for the research that had stirred doubts. In a swift series of steps, Wright continues, the NIH devised the regulations in close collaboration with the Recombinant Advisory Committee (RAC), which included many of those

fears of hazards could now be certified as baseless, that the risks of runaway dangerous microbes were demonstrably not credible. In 1979, those who were regulated reassessed the risks in a stacked committee meeting, disregarding the unknowns in a way that caused *Nature* to observe: "Where were the critics — and there are scientific critics with genuine arguments — to bring matters to a head? To the outside observer, the view that risks are negligible went through on the nod."

The recombinant DNA story has been told before, starting as far back as 1982, with Sheldon Krinsky's durable *Genetic Alchemy* (MIT Press). Wright adds an additional dimension by comparing events in the United States and the United Kingdom, where labour unions representing laboratory workers played a limited role in the regulatory process. The author deplores the absence of similar participation in the United States, although conceding that "the evidence suggests that the unions had little influence" on the formative stages of regula-

tion in the United Kingdom. When they did, she points out, they focused on the safety of laboratory personnel, whereas the debate in the United States swirled around melodramatic epidemic scenarios that those who had been at Asilomar later ridiculed as the work of irresponsible sensationalists. *Molecular Politics*, however, deals mainly with the US experience.

In the end, Wright acknowledges, perhaps a bit sourly, the advocates of deregulation have been vindicated — no known harm has resulted from recombinant DNA research. Why, then, be concerned? The proper answer, it seems to me, is that the benign outcome must be credited to scientific intuition and good luck rather than wise policy and practice. Wright, on the other hand, soars off with assertions that, with regulations gone, “plans are proceeding apace, with few real restraints, to explore chemical and biological warfare applications of genetic engineering and to increase the resistance of agricultural plants to herbicides and produce a large variety of genetically modified plants and microbes for release into the environment”.

Nonetheless, Wright's disparaging assessment of the claims of altruism and scientific responsibility ring all too true in this detailed and well documented work, much of it based on interviews with principal figures and archival materials. But this is a bloated book, far too long for what it delivers. In many sections, the author employs the tactic of outlining what is to come, then delivering the goods and following up with a summary of the preceding pages. The book also contains fortunately limited ventures into sociological patois that the author uses to explain her scholarly strategy. For example:

This study adopts a framework that is both postpluralist and constructivist in orientation. I assume that policy arenas generally bear the imprint of their creators in the form of structural bias that unevenly distributes influence, access, and control of the agenda, and that the effects of bias may be investigated at all the levels of policy formation identified above. Moreover, in contrast to the pluralist position, I assume that interests shaping the policy arena can be defined independently of those effects, by examining their historical formation. This approach can be used to elucidate the most problematic level of “non-decision making” (level IV) identified by postpluralists — the shaping of values and preferences so that some issues are not even formulated.

The bulk of this work, however, is lucid and raises important questions about Next Time, which will surely come from the restless advance of science. □

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Olympian achievements

Jürgen Mittelstraß

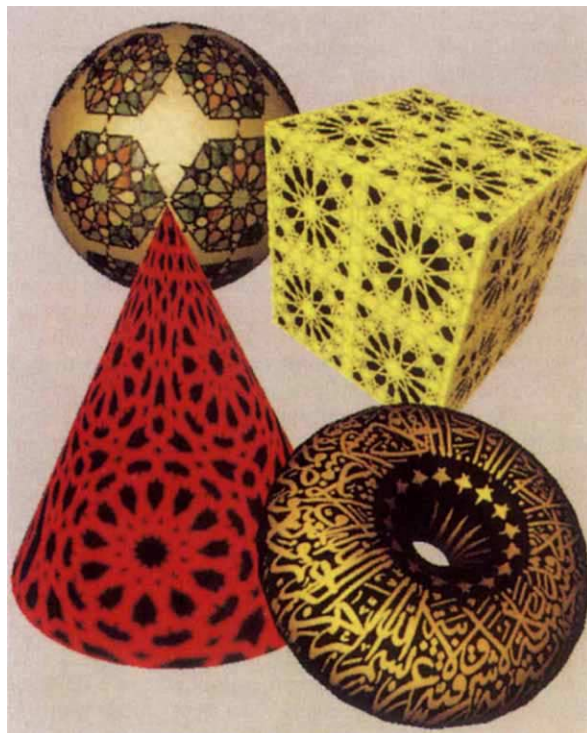
Styles of Scientific Thinking in the European Tradition, 3 Volumes. By A. C. Crombie. Duckworth: 1994. Pp. 2,456. £180, \$270.

HISTORY of science is for many a museum piece exhibited in the temple of science for the amusement and edification of its friends and admirers, but without relevance to science of the present or future. For some, it is a reservoir of case studies used in argument but is not itself a genuine object of knowledge. For others, it remains the expression of the law-like development or transformation of the absolute spirit in its history. None of these is Alistair Crombie's history of science. The first sentence of this gargantuan book makes this quite clear: “This work is a study of the scientific vision explored and controlled by argument and evidence, and their diversification by scientific experience, which constitute the long history of European scientific thought, initiated by the ancient Greeks, in the search for principles at once of nature and of argument itself.”

Crombie began his career as a biologist with work that is still cited, and then made a name for himself as a historian of science with *Augustine to Galileo* (1952) and *Robert Grosseteste and the Origins of*

Experimental Science (1953). He has been president of the British Society for the History of Science and of the *Académie Internationale d'Histoire des Sciences*, Garden Master of Trinity College, Oxford and a recipient of the research prize of the Alexander von Humboldt Foundation. He also has an honorary doctorate from the University of Paris. He has now written his masterpiece — a methodologically orientated history of scientific thought from the Greeks to the Darwinians. Grosseteste and Galileo were the gods to whom Crombie dedicated his earlier work, but now the entire scientific Olympus from the Pre-Socratics to the nineteenth century has been mapped.

Vision, nature and argument are joined together, according to Crombie, in six styles of scientific thinking, which he identifies as follows: postulations, that is, the Greek style founded on the demonstrative power of geometry and arithmetic; the experimental argument, which both controls postulation and explores nature by designed observation and measurement; hypothetical modelling designed as a method of elucidating the unknown properties of a natural phenomenon by simulating it with known properties of artefacts; taxonomy as an explicit logic of classification; probabilistic and statistical analysis, founded on the discovery of the statistical as a new form of regularity; and historical derivation, originally founded as a method of taxonomic and causal analysis and synthesis and later applied to genetic development. Crombie describes and analyses these styles in great depth and



ISLAMIC art provides a marvellous illustration of symmetry, as shown in these computer-generated images based on a calligraphy from the Koran. The picture is taken from *Symmetries of Islamic Geometrical Patterns* by Syed Jan Abas & Amer Shaker Salman, which forges links between the artistic and the scientific, past and present. Published by World Scientific, £27, \$38.

with immense historical knowledge, displaying both their historical and their methodological significance. They can themselves be traced back to the discovery by the Greeks of scientific rationality, that is, to the conception of *theoria* in which the search for principles of nature is combined with the search for principles of scientific argument — a conception that today still, through the long bypaths of European rationality, constitutes the world of science. Crombie presents this long road of development convincingly as the methodical fulfilment of the original Greek conception.

The six styles of scientific thinking do not constitute a historical order in the strict sense, nor are they closed models of rationality with each dominating an epoch; rather, they are different methodical ways of thinking scientifically, ordered both synchronously and diachronously, sometimes incommensurable, usually in combinations, innovative not only on the level of scientific answers but also on the level of scientific questions. What Thomas Kuhn's influential conception has meant for philosophy of science, Crombie's will mean for the history of science; both authors think and write in the same spirit — that of European rationality in which the historical and the systematic (historical origins and rational claims to validity) are joined in the great work of science.

It is impossible to present the wealth of scientific material contained in this book even in part here. We have before us a history of scientific method that meets all the professional standards of modern historiography of science and at the same time displays a kind of learning and scholarship that has now become rare even in philosophy and the humanities. The almost 900 pages of notes and references are a monument to this professionalism and scholarship. They reward the professional historian without getting in the way of the general reader. Crombie is indeed familiar with all scientific styles and has his own impressive style. In the preface he remarks that the book is a celebration of the beauty and elegant economy of scientific thinking, of its combination of creative imagination with precise reasoning, of its enigmatic matching of nature with mathematics and of mathematics with nature, and of its intellectual and practical power for good. But the book is also a celebration of the beauty and elegant economy with which the history of science can be written — if the author happens to be Crombie.

Future historians of science who, with the necessary love for detail and the self-imposed duty to consider the larger picture, attempt to write the history of science, will need great endurance to come as far along the path that Crombie has pursued with such remarkable clarity. And once again, as was the case with

the achievements of a Koyré and a Dijksterhuis, they will stand on the shoulders of a giant. □

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The joy of sex

Alexander H. Harcourt

Human Sperm Competition: Copulation, Masturbation and Infidelity. By R. Robin Baker and Mark A. Bellis. *Chapman and Hall*: 1994. Pp. 353. £45, \$60.

The Difference Between the Sexes. Edited by R. V. Short and E. Balaban. *Cambridge University Press*: 1994. Pp. 479. £55, \$100 (hbk); £19.95, \$29.95.

"HIGGAMUS Hoggamus, Woman is Monogamous; Hoggamus Higgamus, Man is Polygamous." If the epigram is right, then there would, of course, be no sperm competition in humans, and Robin Baker and Mark Bellis could not have written their book. They therefore begin by arguing that women are in fact polyandrous and go on to present all sorts of data on other forms of human behaviour — including that of sperm and the female reproductive tract — that they suggest are adaptations to pervasive sperm competition. Although their views may seem iconoclastic, the immense amount of information, all of it carefully documented methodologically, adds to the development of another area of biology in which anthropologists probably know more about their own species than zoologists do of those they study — fruit-flies perhaps excepted. It is a pity that the authors often give the impression of being deliberately provocative, because I suspect that clinicians as well as their zoologist colleagues would have paid more attention to more gently presented findings and argumentation.

Cambridge University Press's series on "Reproduction in Mammals" is justly famous for its clear and interesting presentation of up-to-date information and theory. *The Difference Between the Sexes* continues in this tradition; indeed, so well done is it that it almost makes for bedtime reading. As with its predecessors, the text contains no references. Instead, a short list of suggested reading is given at the end of each chapter, giving the impression that the book is aimed at undergraduates. Far from it: this is an essential reference for all workers in the field.

The book encompasses the full range of study, from delighted descriptions of

natural history through to biochemistry, physiology, anatomy and behaviour. Proximate, developmental, functional and evolutionary levels of explanation are all covered, sometimes in a single chapter. Short's opening overview starts with Darwin's *The Descent of Man and Selection in Relation to Sex*; continues with sexual-selection theory by way of Fisher's *The Genetical Theory of Natural Selection*; moves on to sex determination and how the amazing early contrast between germ cells in mitotic and meiotic behaviour depends on their complement of sex chromosomes and genes; and closes with the influence of originally parasitic mitochondrial DNA on the oocyte and the origins of sex differences in gamete size.

SPL

IMAGE UNAVAILABLE FOR COPYRIGHT REASONS

Winner takes it all — human sperm on inner surface of the uterus.

The rest of the book is similarly eclectic, with chapters on the environmental influence of sexual development (depending on the species, nutrition, pH, temperature or sex of associates can influence gender); costs of sex (metabolically active, large males of polygynous species, and their mothers, suffer); sexual dimorphism in primates (large males, with their greater competitive ability, have not been favoured, as we all thought; rather, small females have been favoured because of their higher reproductive rate); and bizarre sex-determining mechanisms of some rodents (in one species, XY animals turn out to be females, which give birth only to daughters, and in another, two sexes clearly exist, and yet both are XO — the way in which gender is determined is still unknown, despite nearly half a century of research).

The incorporation of several levels of explanation and the clarity of writing are epitomized in Wingfield's chapter on hormones, behaviour and mating systems

of birds. There are good functional arguments why we should expect a trade-off between competition for mates and investment in offspring. It turns out that there is also a trade-off in behavioural responses of birds to artificially boosted testosterone levels: the increased competitiveness of males suppresses paternal care, so that the monogamous males that have been made polygynous have lowered reproductive success, despite more females in their larger territories. In an equally fitting match, sex differences in peak levels of testosterone in plasma correlate across taxa with sex differences in morphology and behaviour — in monogamous species. In polygynous species, males have far higher peaks than females, however great the behavioural or anatomical differences; among monogamous species, by contrast, the often highly competitive females of monomorphic species nearly match the males in hormone level. An extreme example is the Californian Santa Barbara Island population of western gulls in which a severe shortage of males (perhaps caused by feminizing pollutants) correlates with intense competition among females for mates and with a rise in female testosterone levels to match male levels.

As well as addressing the evolutionary and functional 'why?' questions and the physiological and biochemical 'how?' questions, the editors wanted simply to describe the differences between the sexes. And throughout the book, a love of natural history is obvious, in both the illustrations and the writing. "Reproduction is a symphony in physiology", writes Crews, an evocative alternative title for a paper he refers to, which deals with the "integration of internal and external stimuli in the regulation of lizard reproduction". Some of the sex differences may astonish. As Gustafsson says: "Although it may not be generally recognized among scientists interested in sex differentiation, many metabolic events in rodent liver are sexually dimorphic". What's more, the proximate switch that determines the gender of the liver is not the nature of the sex steroids reaching it, as we might expect, but the schedule of release of growth hormone from the pituitary: oestrogens change the pulsatile 'male' release of hormone to a steadier 'female' release, which converts the default male liver to a female one. At the same time, the picture, in a chapter on hymenopteran sexual dimorphism, of one tiny caste of female worker ant *Pheidologeton diversus* standing on the head of a giant caste, whose eye is as large as the smaller ant's head, reminds us that there are also huge differences within the sexes.

Balaban comments on the past sexist ways of orchestral societies and remarks with obvious approval that the sex ratios

of performers "have made significant progress towards equality in the last two decades". Although the 2:1 ratio of male to female authors (3:1 ratio of first authors) of the 22 chapters in this book are certainly better than those of most edited science volumes, we clearly still have a long way to go to remove this particular difference between the sexes. □

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Some gentlemen prefer bonds

Wilfred Niels Arnold

Blondes in Venetian Paintings, the Nine-Banded Armadillo, and Other Essays in Biochemistry. By Konrad Bloch. Yale University Press: 1995. Pp. 261. \$30, £17.95.

DISTINGUISHED scientists, having demonstrated their intellectual ability, need no further justification for publishing their thoughts and experiences beyond their notable contributions. Such extraordinary writing has included accounts of the steps leading up to a famous discovery; documentaries of the life and times of the author (usually with some attention paid to problems, diversions and lucky breaks); and explorations of entirely new fields ranging from the poetic to the politically sensitive. Konrad Bloch's collection of essays is none of the above; it is closer to an older genre, in the tradition say of John Tyndall's *Fragments of Science for Unscientific People: A Series of Detached Essays, Lectures and Reviews*, published in 1871.

In his modest prefatory remarks, Bloch claims no special background and eschews autobiography. But the sensitive dedication to the late Rudolph Anderson, who aided the author's immigration to the United States, and the occasional professional reference to a mentor, colleague or student are among the most engaging items, and whet one's appetite for more.

Eight of the thirteen essays deal directly with chemicals. There is much discussion about the relative fitness of selected molecules for the biological environment — chemical evolution in the Darwinian sense. The pieces dealing with cholesterol and related compounds are understandably the most authoritative. (For his work on cholesterol, Bloch shared the 1964 Nobel prize for physiology or medicine with Feodor Lynen.)

The author displays a wide appreciation of metabolism, nutrition and comparative biology. The essays vary greatly

in originality and new insights, although they all have something of interest for a diverse readership, as intended. Sometimes the grand scale falls short, as in the piece that explores reasons why insects have a disaccharide, trehalose, in their haemolymph, whereas animal blood contains a monosaccharide, glucose. Most green plants translocate another disaccharide, sucrose, but this is not even mentioned (working hypotheses for the selection of all three translocates have been published). There are other instances where Bloch seems not to bother to bring his chosen topic to a completely satisfying endpoint.

The catchy lead in the book's title is the subject of the first essay. The author wonders about the high incidence of blondes in fifteenth- and sixteenth-century masterpiece paintings in Venice, where the native population was decidedly brunette. He masterfully and entertainingly sets up working hypotheses like straw men and then knocks them over with scientific logic. One of his art-historian friends then directs him to seemingly definitive answers in nineteenth-century treatises from France and Italy. This proves to be something of an anticlimax, although the chemistry remains to be solved. Possible mechanisms are offered, all reasonable but never tested, and the reader starts the next essay slightly bewildered as to why the old Italian recipes, which are reproduced in this book, have not yet been tried out at Harvard University, the author's alma mater.

The production quality of the book is modest, incorporating only one black-and-white illustration, and there are a few inexcusable misrepresentations in the depictions of some amino-acid structures.

The author includes several interesting asides; in a footnote, for example, he tells of the financial remuneration he received for his first scientific publication, a revelation that will both surprise and titillate young investigators now accustomed to exorbitant page charges by some journals. A tiny sortie into the misnomer of molecular biology is delivered with restraint but our chemist leaves no doubt about where the emphasis should fall in biochemistry. One is left with the overall impression that Bloch has much more to say. Nevertheless, any reader with a general background in science should find in these essays many fascinating items. □

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■ The fifth edition of the classic textbook *Chemical Thermodynamics: Basic Theory and Methods* by Irving M. Klotz and Robert M. Rosenberg was recently published by Wiley at \$54.95.

Principles of CDK regulation

David O. Morgan

As key regulators of the cell cycle, the cyclin-dependent kinases must be tightly regulated by extra- and intracellular signals. The activity of cyclin-dependent kinases is controlled by four highly conserved biochemical mechanisms, forming a web of regulatory pathways unmatched in its elegance and intricacy.

THE major transitions of the eukaryotic cell cycle are triggered by the cyclin-dependent protein kinases (CDKs, reviewed in refs 1–4). To ensure the proper timing and coordination of cell cycle events, CDK activity is tightly controlled by several complex mechanisms. In simple systems such as the early frog embryo, CDK activity is driven by a biochemical oscillator that is independent of the events it induces. In somatic cells and yeast, additional regulatory layers have evolved to ensure that changes in CDK activity (and thus the initiation of cell-cycle events) are delayed when growth conditions are suboptimal or when preparations for the next cell cycle event are incomplete. Thus in most cells the CDK oscillator, although retaining some autonomy, is influenced by an array of intra- and extracellular information.

Cellular CDK levels tend to remain in constant excess throughout the normal cell cycle, and regulation of catalytic activity is primarily post-translational (Fig. 1). CDK activation requires cyclin binding and phosphorylation of a conserved threonine by the CDK-activating kinase (CAK). The active CDK–cyclin complex can be inhibited by phosphorylation of a conserved threonine–tyrosine pair or binding to CDK inhibitory subunits (CKIs). The biochemical principles underlying these control systems are now superficially understood, and many of the protein components that regulate CDKs have been identified.

The CDK catalytic subunit

What defines a CDK? CDKs are closely related in size (35–40K) and sequence (>40% identity), and associate with and are activated by a cyclin regulatory subunit. The definition of CDKs does not include limits on biological function: although most known CDKs are involved in cell-cycle control, the list of CDKs involved in other processes is growing^{5–8}.

Many protein kinases are closely related to the CDKs, but relatively few have been shown to possess cyclin-dependent activity. In the fission yeast *Schizosaccharomyces pombe*, a single major CDK has been identified (Cdc2); in the budding yeast *Saccharomyces cerevisiae*, the major CDK, CDC28, has recently been joined by a second family member, PHO85^{9,10}, and a third candidate, KIN28, is under investigation¹¹. In human cells, the growing list of CDKs now includes the founding member, CDC2 (or CDK1), and CDK2 to CDK7. Our understanding of CDK structure and function is based largely on studies of the prototypical CDKs of *S. pombe* (Cdc2), *S. cerevisiae* (CDC28), and vertebrates (CDC2 and CDK2). It is not clear if the details of their regulation are conserved throughout the family.

The typical CDK catalytic subunit contains a 300 amino acid catalytic core that is completely inactive when monomeric and unphosphorylated. The crystal structure of the human CDK2 apoenzyme shows that it is held in an inactive state by at least two major structural restraints^{12,13}: first, the substrate binding site is blocked by an extended loop termed the T loop; and second, side chains in the ATP-binding site are oriented so that the ATP phosphates are poorly positioned for efficient phosphotransfer. As yet, nothing is known about the conformational

changes that accompany CDK2 activation by cyclin binding and phosphorylation.

CDK activation by cyclin binding

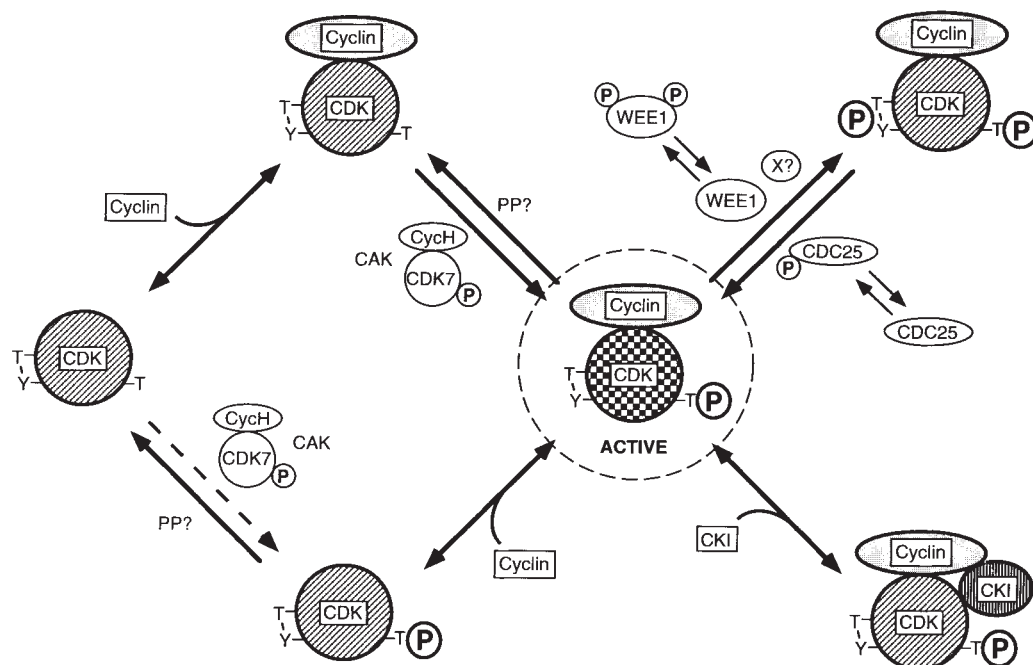
The primary regulator of CDK activity is the cyclin subunit. Originally defined as proteins whose levels oscillate during the cell cycle, cyclins are now more accurately defined as members of a family of structurally related proteins that bind and activate CDK catalytic subunits. Homology among cyclins is often limited to a relatively conserved domain of about 100 amino acids, the cyclin box, which is responsible for CDK binding and activation^{14,15}. Mutations in this region inhibit both binding and activation, suggesting that the two functions are not easily dissociated. Each CDK interacts with a specific subset of cyclins, although the size of this subset varies. For example, CDC28 associates with many different cyclins (nine at last count)², whereas human CDC2 interacts with relatively few. In addition, a single mammalian cyclin can sometimes interact with multiple CDKs⁴. Our understanding of the mechanics of CDK–cyclin binding remains limited, as no detailed analyses of binding kinetics have been done.

The cyclin family tree has recently been stretched by studies of CDK5, a protein kinase thought to play a role in neural development^{7,8}. Activation of CDK5 requires association with a protein known as p35, which apparently lacks the conserved pattern of amino acids normally found in cyclins. p35 may thus be the founding member of a new class of CDK regulatory subunits.

Cyclin function is primarily controlled by changes in cyclin levels, which increase characteristically at specific cell cycle stages; indeed, cyclins are often categorized by the stage at which they are expressed. In the early *Xenopus* embryo, oscillations in the mitotic cyclin, cyclin B, are produced mainly by changes in the rate of cyclin degradation. Constant synthesis during the cell cycle results in a roughly linear increase in cyclin concentration, until at mitosis cyclin degradation abruptly increases, producing a rapid decline in cyclin levels. Cyclin degradation probably involves the ubiquitin-dependent proteolytic machinery, and requires a small sequence motif (the destruction box) near the amino terminus of mitotic cyclins¹⁶. The trigger for destruction may be the increased CDC2 activity at mitosis, and there is evidence that components of the ubiquitin system are activated by CDC2¹⁷.

In somatic vertebrate cells and yeast, cyclin levels are also controlled at the transcriptional level. In mammalian cells, sequential oscillations in the levels of the major cyclins (E, A and B) largely reflect messenger RNA levels^{18–20}, but little is known about the mechanisms that generate these waves of gene expression. Much more is known in *S. cerevisiae*, where an elegant transcriptional programme governs cyclin expression. Activation of CDC28 by the G1 cyclins (CLNs) leads to activation of *CLN1* and *CLN2* transcription in a positive feedback loop (see ref. 2 for review). Activation of CDC28 by CLNs also inactivates the machinery responsible for the degradation of the CLB

FIG. 1 Principles of CDK regulation. At centre right lies the active Thr 160/161-phosphorylated CDK-cyclin complex. Moving outward along any of the four pathways results in inactivation; moving inward leads to activation. This is a highly simplified scheme from which many pathways and alternative states have been omitted; see text for details.



mitotic cyclins²¹, allowing CLB levels to rise after G1. CLB proteins then help to repress *CLN* transcription, while at the same time stimulating their own transcription in another positive-feedback loop²². The resulting increase in CLB levels leads to mitotic activation of CDC28, which then triggers CLB destruction.

Cyclins are thought to contain regions that target the CDK to specific substrates or subcellular locations. In addition to simply activating the associated CDK, they can thus promote activity toward specific substrates²³⁻²⁵. The enhanced activity of certain complexes is probably due to positive interactions between the cyclin and the substrate²³⁻²⁵.

CDK activation by phosphorylation

In addition to cyclin binding, complete CDK activation requires phosphorylation at a conserved threonine residue (Thr 161 in human CDC2, Thr 160 in CDK2). In CDK2, Thr 160 lies in the T loop that blocks the protein substrate binding site; its side chain is inaccessible to solvent, suggesting that the loop must move to allow phosphorylation^{12,13}. Phosphorylation may affect the cyclin binding site, as it enhances the binding of some CDK-cyclin pairs^{26,27}; conversely, cyclin binding may enhance phosphorylation.

Considerable effort has recently been devoted to the characterization of CAK, the enzyme responsible for the phosphorylation of Thr 160/161. CAK is a multi-subunit enzyme whose catalytic subunit is a highly conserved, CDK-related protein kinase termed MO15²⁸⁻³⁴. The second major subunit of human CAK is a new cyclin, termed cyclin H (refs 33, 35). Addition of purified cyclin H to MO15 *in vitro* reconstitutes CAK activity, demonstrating that CAK, like its substrates, is a CDK-cyclin complex³³. MO15 has therefore been renamed CDK7.

CDK7, like its substrates, contains a potential phosphorylation site in the T loop (Thr 170 in human CDK7), and mutation of this residue greatly reduces kinase activity^{32,33}, indicating that activation may require phosphorylation at this site. CAK may also be regulated by additional subunits: mammalian CAK appears to contain a third subunit that is slightly smaller than cyclin H^{31,33}.

Starfish and *Xenopus* CDK7, like the mammalian CDK7-cyclin H complex, can readily phosphorylate both CDC2 and CDK2 complexed with various cyclins^{28-30,33}, and the mammalian enzyme also phosphorylates the more distantly related

CDK4³⁴. Thus, a single CAK can activate all of the major CDK-cyclin substrates involved in vertebrate cell-cycle control.

During the normal vertebrate cell cycle, phosphorylation of Thr 160/161 tends to rise and fall in parallel with cyclin binding^{36,37}. Changes in phosphorylation are probably not due to changes in CAK activity, which does not appear to change during the cell cycle^{31,32,34,38}, but appear to reflect the ability of cyclin binding to stimulate CDK phosphorylation. CAK activity is therefore not rate-limiting during normal cell proliferation, although its regulation may be important under some growth conditions.

CDK inhibition by phosphorylation

There are many ways to inactivate a CDK-cyclin complex. Two of the most obvious are to remove the cyclin or dephosphorylate Thr 160/161. The importance of Thr 160/161 dephosphorylation is uncertain at present, but decreased synthesis or increased degradation of cyclins are clearly important *in vivo*.

CDK-cyclin complexes can also be inhibited by phosphorylation at two sites near the amino terminus (Thr 14 and Tyr 15 in human CDC2 and CDK2). In CDK2, the side chains of these residues hang from the ceiling of the ATP-binding site and are certainly in a position to affect kinase activity when phosphorylated¹². The mechanism of inhibition is unknown, but phosphorylation of Tyr 15 does not appear to inhibit ATP binding³⁹. Both residues are buried beneath the T loop and are inaccessible to solvent, suggesting that the T loop must be moved to allow phosphorylation. This may be accomplished by cyclin binding, as phosphorylation is thought to be cyclin-dependent^{39,40}.

Phosphorylation of Thr 14 and Tyr 15 is particularly important in the control of CDC2 activation at mitosis. Like Thr 161 phosphorylation, phosphorylation of Thr 14 and Tyr 15 roughly parallels the rise in cyclin B levels that occurs as cells approach mitosis^{36,40}. CDC2-cyclin B complexes are thus maintained in an inactive state, until Thr 14-Tyr 15 dephosphorylation at the end of G2 activates CDC2. This abrupt dephosphorylation is brought about by coordinated changes in the activities of kinases and phosphatases acting at these sites.

The major candidate for the Tyr 15 kinase is Wee1, originally identified in *S. pombe*. *In vitro*, Wee1 can phosphorylate peptide substrates on both threonine and tyrosine residues, leading to speculation that it might be a dual-specificity kinase capable

of phosphorylating both Thr 14 and Tyr 15. However, Wee1 phosphorylates CDC2 *in vitro* at Tyr 15 but not Thr 14, supporting the existence of a separate Thr 14 kinase^{41,42}. Thr 14 kinase activity has recently been detected in extracts of *Xenopus*⁴³ and human cells⁴⁴. Intriguingly, the *Xenopus* activity is associated with membranes and can be separated from a Tyr 15-specific kinase activity in the soluble fraction⁴³. The identity of these enzymes and their relationship to Wee1 will be of great interest.

Wee1 activity declines during mitosis, contributing to the fall in inhibitory phosphorylation at this stage. Decreased activity during mitosis is due to phosphorylation of Wee1, although the kinases responsible are unclear and may vary in different species. In fission yeast, the protein kinase Nim1 inhibits Wee1 by phosphorylating its C-terminal catalytic domain⁴⁵⁻⁴⁷. Higher eukaryotic homologues of Nim1 have not been found, but *Xenopus* extracts contain a kinase activity that inhibits Wee1 by phosphorylating its N-terminal domain⁴⁸.

Thr 14 and Tyr 15 are both dephosphorylated by a dual-specificity phosphatase termed CDC25. CDC25 activity increases during mitosis, largely because of increased phosphorylation in its N-terminal half (see ref. 49 for review). During mitosis, the kinase responsible for this phosphorylation is activated and the corresponding phosphatase is inhibited. The CDC25-stimulatory kinase may be CDC2 itself^{23,50}, forming the basis of an elegant positive-feedback system that could in theory induce the abrupt mitotic dephosphorylation of CDC2. Positive feedback may also be achieved by coordinated effects on other components: CDC2 may stimulate the kinase that inactivates Wee1 and inhibit the phosphatase(s) that inactivates CDC25 and activates Wee1 (see ref. 49 for review).

CDK inhibition by inhibitory subunits (CKIs)

The fourth major mechanism for CDK regulation involves a diverse family of proteins, termed the CKIs, that bind and inactivate CDK-cyclin complexes. At present, seven family members have been clearly identified. Two CKIs from *S. cerevisiae*, FAR1⁵¹⁻⁵³ and p40 (SIC1/SDB25)⁵⁴⁻⁵⁶, inhibit CDC28-CLN and CDC28-CLB complexes, respectively. A third yeast CKI (PHO81) inhibits the activity of PHO85-PHO80, a CDK-cyclin complex involved in phosphatase gene expression⁶. The four major mammalian CKIs fall into two classes: p21 (CIP1/WAF1/CAP20/SDI1)⁵⁷⁻⁶¹ and p27 (KIP1)⁶²⁻⁶⁴ are related proteins with a preference for CDK2- and CDK4-cyclin complexes, whereas p16^{INK4} and p15^{INK4B} are closely related CKIs specific for CDK4- and CDK6-cyclin complexes^{65,66}.

Our knowledge of CKI inhibitory mechanisms remains sketchy. Most CKIs bind tightly to Thr 160/161-phosphorylated CDK-cyclin complexes and directly inhibit kinase activity. In many cases (FAR1, p40, p21), CKIs are phosphorylated by their CDK target, suggesting an interaction with the protein substrate binding site^{53,54,67}. Studies of p40 indicate that it reduces both the V_{max} and the K_m of CDC28 for protein substrate⁵⁴. The stoichiometry of CKI-CDK binding is unclear: in the case of p21, there is evidence that two CKI subunits must bind to inhibit the activity of each CDK2 catalytic subunit⁶⁷. Whereas most CKIs apparently recognize CDK-cyclin complexes rather than CDK monomers, p16^{INK4} associates with CDK4 monomers *in vivo*⁶⁵, and may be capable of blocking cyclin binding.

How are CKIs regulated? For p21, a major mode of regulation is transcriptional. p21 transcription is induced by p53, a transcriptional regulator that mediates cell-cycle arrest following DNA damage^{68,69} and in senescence⁶¹. Significant levels of p21 also exist in actively proliferating human⁶⁷ and mouse fibroblasts⁶⁰; these basal p21 levels may provide a threshold that must be exceeded before complexes can become active. Transcriptional control may also be important for p15^{INK4B}, whose expression in human keratinocytes is greatly enhanced by treatment with the negative growth factor TGF β ⁶⁶.

The second member of the p21 family, p27, may be regulated in some cells by an unusual post-transcriptional mechanism. p27

was originally identified as a CDK2 inhibitor whose activity increases in mink epithelial cells treated with TGF β ⁶². The total amount of p27, however, is unaffected by TGF β treatment or during the cell cycle^{63,64}. Rather, p27 appears to be sequestered in a heat-labile compartment, from which it is released upon TGF β treatment. In other cases, such as the G1 arrest induced by cAMP treatment of macrophages, total cellular levels of p27 are regulated⁷⁰. p27 may also be involved in the actions of positive growth factors. Upon stimulation of the antigen receptor, T cells progress from quiescence to G1, and must be further stimulated by interleukin-2 (IL-2) to progress into S phase. IL-2 stimulation appears to induce proliferation by decreasing levels of a CDK2 inhibitory activity that is probably p27⁷¹.

Complex mechanisms also govern the action of FAR1, which inhibits CDC28-CLN complexes in G1 yeast cells treated with the mating pheromone α -factor. Mating pheromone initiates a signalling cascade that leads to the phosphorylation of FAR1 by the protein kinase FUS3; this phosphorylation may be required to induce FAR1 binding to CDC28-CLN complexes, resulting in their inhibition (and G1 arrest)^{52,53}. α -Factor also stimulates FAR1 transcription⁵¹. In cycling cells, FAR1 transcription and protein levels peak in early G1⁷², increasing levels of the protein at the appropriate stage.

Like cyclins, CKIs can also be regulated by stage-specific degradation. In yeast, p40 binds and inhibits CDC28-CLB5 in G1, until it is degraded by the ubiquitin-dependent proteolysis machinery at the G1/S transition⁷³; this allows the accumulation of active CDC28-CLB5 complexes, which initiate S phase.

Future directions

Four major CDK regulatory mechanisms have been identified together with a large inventory of CDK regulators. There is still much to be learned about how these components form a highly responsive regulatory network. Complete understanding of this network will require quantification of the reaction kinetics and binding interactions involved in CDK regulation. Ideally, this information should be accompanied by detailed structural analyses of the components in various states of activity and assembly.

New layers of CDK regulation undoubtedly remain to be discovered. Some regulation probably involves subcellular localization: CDKs and their regulators are found in specific locations, but we know little about the mechanisms responsible or whether localization is regulated. One of the enduring mysteries of CDK regulation is the role of the fission yeast protein, p13^{suc1}, and its homologues, the CKS proteins, in budding yeast and vertebrates (see ref. 74). These small proteins, which are essential for cell-cycle progress in yeast, have long been known to bind tightly to certain CDKs *in vivo*. They do not, however, have any clearcut effect on CDK function *in vitro*. The crystal structure of one family member, CksHs2, suggests that these proteins may be involved in CDK oligomerization in the cell⁷⁴.

We are just beginning to appreciate the connections between CDK regulation and cancer. Overexpression of certain cyclins, particularly those of the D class, can contribute to cell transformation⁷⁵ and CKIs have also been identified as potential tumour suppressors. The p16 gene, in particular, is lost in a majority of tumour cell lines and in a considerable number of primary tumours (see refs 76, 77 for review). Studies of CDK regulation in cancer, accompanied by a basic understanding of CDK enzymology, may eventually yield new methods for reversing the transformed phenotype. □

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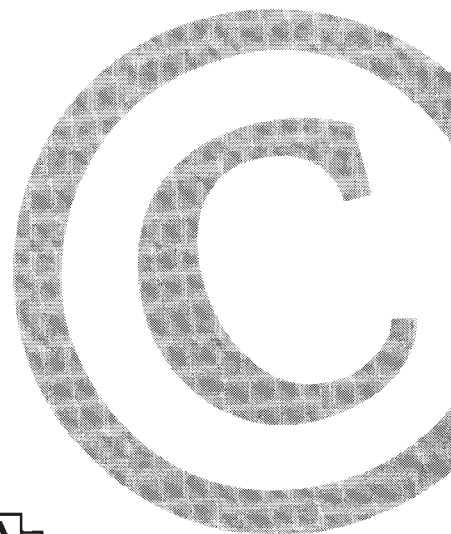
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The G-protein-gated atrial K⁺ channel I_{KACH} is a heteromultimer of two inwardly rectifying K⁺-channel proteins

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Heart rate is slowed in part by acetylcholine-dependent activation of a cardiac potassium (K⁺) channel, I_{KACH}. Activated muscarinic receptors stimulate I_{KACH} via the G-protein βγ-subunits. It has been assumed that the inwardly rectifying K⁺-channel gene, *GIRK1*, alone encodes I_{KACH}. It is now shown that I_{KACH} is a heteromultimer of two distinct inwardly rectifying K⁺-channel subunits, *GIRK1* and a newly cloned member of the family, *CIR*.

ACETYLCHOLINE (ACh) secreted from the vagus nerve binds cardiac muscarinic receptors (m2) to slow heart rate. This is accomplished in part by pertussis-toxin-sensitive G-protein gating of inwardly rectifying potassium channels (I_{KACH}). Purified¹ or recombinant² G_{βγ} subunits activate I_{KACH} when applied to the internal surface of isolated membrane patches containing the channel. A complementary DNA (*GIRK1*) presumed to encode the channel has been cloned by homology³ and expression⁴ methodologies. *GIRK1* is a member of a gene family encoding inwardly rectifying K⁺ channels. When expressed in *Xenopus* oocytes, the protein encoded by *GIRK1* displays many of the properties of I_{KACH} first defined at the single-channel level in cardiac cells⁵, including single-channel conductance and Mg²⁺-dependent inward rectification³. However, there remain unexplained differences in its behaviour, including a high level of agonist-independent channel activity, relatively weak stimulation by G proteins (compare ref. 3 with refs 1, 2) and strong activation via coexpressed β-adrenergic receptor⁶. Reasoning that these discrepancies may be due to an unidentified subunit or unknown regulatory elements missing in the oocyte expression system, we used antibodies directed against the predicted *GIRK1* protein to probe the nature of atrial *GIRK1*. Here we report that a protein homologous to members of the inwardly rectifying K⁺-channel family coimmunoprecipitates with atrial *GIRK1*. This protein, which we have termed CIR (cardiac inward rectifier), was recently reported to generate ATP-sensitive channels (I_{KATP}; ref. 7). We present evidence that CIR forms a unique K⁺ channel when expressed in *Xenopus* oocytes, Sf9, CHO and HEK cells, but does not seem to constitute I_{KATP} by itself. When CIR is coexpressed with *GIRK1* in oocytes or CHO cells, G-protein-gated inwardly rectifying K⁺ current is significantly increased and shows properties similar to those of atrial I_{KACH}. We propose that atrial I_{KACH} is a heteromultimer comprised of at least *GIRK1* and CIR polypeptides.

The oligomeric structure of I_{KACH}

In vitro translation of *GIRK1* yielded a product of relative molecular mass 56K (the open reading frame of *GIRK1* cDNA predicts a 56.6K product). Translation in the presence of microsomes yielded a ~58K band in addition to the 56K product, reflecting post-translational modification of *GIRK1* (Fig. 1a). To study native *GIRK1* protein, we generated three different anti-peptide antibodies to putative cytoplasmic domains of the predicted *GIRK1* polypeptide. Two antibodies were developed

against separate 10-amino-acid peptides found within the putative cytoplasmic amino- and carboxy-terminal domains (aNp and aCp, respectively) and the third (aCsh) was raised against the 156-amino-acid C-terminal domain specific for *GIRK1* (this segment has little sequence homology to other known members of the inwardly rectifying K⁺-channel family). All three affinity-purified antibodies immunoprecipitated *GIRK1* translated *in vitro* (Fig. 1a). Antibody aCsh specifically recognized *GIRK1* expressed in *Xenopus* oocytes injected with *GIRK1* messenger RNA, as well as *GIRK1* in Sf9 cells infected by a recombinant *GIRK1* baculovirus (Fig. 1a). In addition, aCsh detected a 58K protein and labelled a broad band between 67K and 72K on western blots of atrial plasma membrane (Fig. 1b). Proteins in the broad 67–72K band represent heavily glycosylated forms of *GIRK1*, as treatment with *N*-glycosidase F completely converted these forms into the 58K species (Fig. 1c). Antibody aCsh did not recognize ventricular proteins on western blot and immunoprecipitated only trace amounts of a 58K protein (Fig. 1b), consistent with the lack of functional I_{KACH} expression in cardiac ventricle. To determine if other proteins associate with *GIRK1*, we immunoprecipitated atrial *GIRK1* with aCsh (Fig. 1b, d). An additional protein product of apparent *M*_r 45K (p45) coimmunoprecipitated with the 58K and 67–72K proteins (Fig. 1b, d). We used the two other anti-peptide antibodies to confirm the specificity of antibody recognition of atrial proteins and their relation to *GIRK1*. Both aNp and aCp immunoprecipitated the same set of 45K, 58K and 67–72K atrial membrane proteins. Furthermore, antigenic peptides completely blocked the immunoprecipitation of all proteins (Fig. 1d).

Using immobilized aCsh, we purified p45 by immunoaffinity chromatography. After enzymatic and cyanogen bromide cleavage, we microsequenced two peptide fragments, DYIPIATDR and FTPVLTLEK (single-letter amino-acid code), which are identical to predicted sequences in a member of the inwardly rectifying K⁺-channel family (CIR; Genbank accession number L35771; Fig. 2a). Given the relatively high homology between members of the inwardly rectifying K⁺-channel family (CIR and *GIRK1* are 57% identical overall), it was possible that CIR was directly immunoprecipitated with the anti-*GIRK1* antibodies. However, the *GIRK1* and CIR proteins have only 28% identity within the domain used to raise the aCsh antibody, and no homology in the regions to which anti-peptide antibodies were made. Furthermore, in control experiments aCsh did not immunoprecipitate CIR translated *in vitro*. These results suggested that atrial I_{KACH} is a complex comprised of at least two different polypeptides, *GIRK1* and CIR.

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The CIR clone

We cloned *CIR* by screening a neonatal rat atrial cDNA library with *IRK1* under low stringency conditions. The resulting clone was termed cardiac inward rectifier (*CIR*) based on its sequence similarity to other members of this family (Fig. 2a, e) and its electrophysiological characteristics. *In vitro* translation of the *CIR* cDNA clone produced a protein of 45K (Fig. 2b). In the presence of canine microsomes, the mobility of CIR protein in the gel was unaltered despite the consensus glycosylation site in the predicted protein sequence (Fig. 2b). An anti-peptide antibody to predicted amino acids 396–409 specifically recognized the 45K protein in membranes of Sf9 cells infected with a recombinant CIR baculovirus (CIR.bac; Fig. 2c). Northern blot analysis of rat multiple tissue blots probed with CIR under high stringency conditions show that CIR is most abundant in heart tissue (not shown). Within the heart, CIR protein is found predominantly in atria (Fig. 2d).

Acetylcholine evoked a small (100 nA) increase in current in *Xenopus* oocytes coinjected with CIR and muscarinic m2 receptor mRNA (Fig. 3a), suggesting that CIR currents may be stimulated by G-protein activation. The current was blocked by 100 μ M Ba²⁺. Oocytes injected with m2 receptor mRNA alone showed no ACh-mediated increase in K⁺ current.

Inasmuch as initial attempts to express CIR in *Xenopus* oocytes produced only nominal currents, alternative expression systems were used. Patch-clamp analysis of Sf9 cells infected with CIR.bac showed an unusual channel current of apparent variable amplitude and short open times (spiky) in 35 out of 37 patches (94%; Fig. 3b). No similar current was observed in control Sf9 cells ($n=10$) or Sf9 cells infected with a β -galactosidase-producing virus ($n=7$). In Sf9 cells infected with CIR.bac, patch

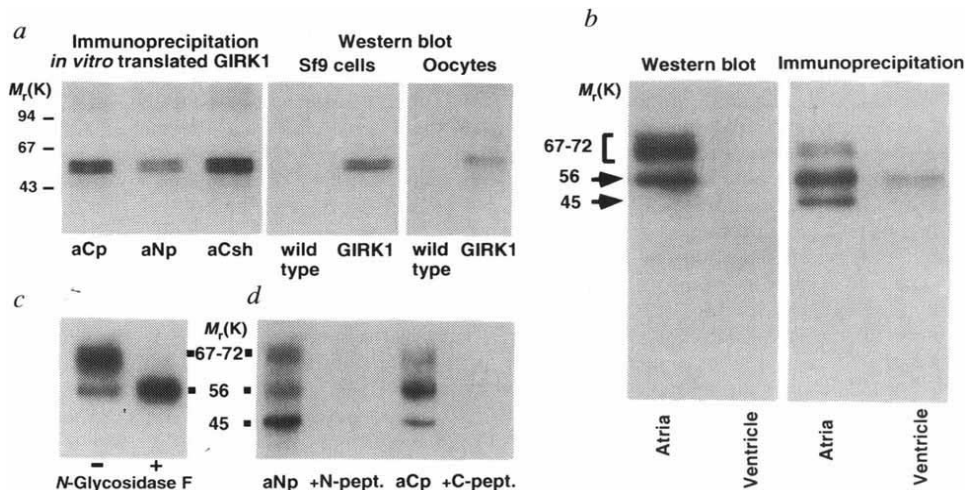
excision (inside-out) resulted in rapid rundown of channel activity. Subsequent addition of 200 μ M GTP- γ S significantly increased channel activity. The presence of 1 mM ATP in the bath did not effectively change the level of activation of GTP- γ S, nor was the effect of GTP- γ S seen in mock-infected Sf9 cells.

We developed a reliable transient expression system in CHO and HEK-293 cells to study inward rectifier clones further. CHO cells were used preferentially because of their relatively low endogenous channel activity under our assay conditions. To improve the chances of assaying only transfected cells, an expression construct containing the murine T-lymphocyte-specific surface protein, CD4, was included in all transfections. Cells expressing CD4, determined by binding of fluorescent anti-CD4 antibodies, were assayed for single-channel currents. Inside-out patches from both CHO (Fig. 3c) and HEK cells transfected with CIR contained currents similar to those observed in Sf9 cells (26/29 patches and 9/12 patches, respectively), but in none of 10 control cells of each line. Channels were active in the cell-attached configuration and ran down in the inside-out patch configuration. The addition of GTP- γ S or ATP to inside-out patches from CHO cells weakly stimulated the channels, but the combination of ATP (100 μ M) and GTP- γ S (10 μ M) elicited significantly more channel activity (Fig. 3c). The non-hydrolysable ATP analogue AMP-PNP (up to 1 mM) did not mimic ATP in this experiment, suggesting that the ATP effect is mediated by phosphorylation rather than simple nucleotide binding. In addition, preliminary results indicate that G_{βγ} stimulates CIR in a manner similar to GTP- γ S. HEK-expressed CIR channels showed similar stimulation by GTP- γ S (data not shown).

Analysis of single-channel histograms from Sf9, CHO and HEK expression systems revealed the non-quantal nature of CIR

FIG. 1 Atrial GIRK1 associates with a 45K protein. a, Specificity of anti-GIRK1 antibodies. Three antibodies (aCsh, aNp, aCp) directed against regions in the N- and C-terminal domains of the predicted GIRK1 protein immunoprecipitated a 56–58K doublet of *in vitro*-translated GIRK1. aCsh recognized recombinant GIRK1 protein heterologously expressed in *Xenopus* oocytes (10 μ g of membrane proteins) and Sf9 cells (2 μ g of membrane proteins) infected with a recombinant GIRK1 baculovirus. b, aCsh antibody recognized a ~58K protein and a broad band of 67–72K on a western blot of bovine atrial plasma membrane proteins (5 μ g). aCsh immunoprecipitated the same set of atrial plasma membrane proteins, and coimmunoprecipitated an additional protein with an apparent M_r of 45K (p45). In contrast, aCsh did not recognize proteins on a western blot and immunoprecipitated only traces of a 56K protein from bovine ventricle plasma membranes. c, The 67–72K proteins recognized by aCsh in atrial plasma membranes are glycosylated forms of atrial GIRK1 protein. Treatment of atrial plasma membrane proteins with N-glycosidase F converted 67–72K proteins into a ~58K form. d, Anti-GIRK1 antibodies (aNp and aCp) raised against N- and C-terminal peptides from GIRK1 immunoprecipitated the same set of proteins (45K, 58K, 67–72K) from atrial plasma membrane as did aCsh. Immunoprecipitation and coimmunoprecipitation were blocked by corresponding antigenic peptides (20 μ M).

METHODS. aNp and aCp antibodies were raised against synthetic peptides (Np, Lys 7–Thr 16; Cp, Gly 445–Lys 454) conjugated to keyhole limpet haemocyanin (KLH), and were affinity-purified. aCsh antibody was raised against a β -galactosidase–GIRK1 fusion protein containing GIRK1 residues 346–501 and purified as described¹¹. *In vitro* translation experiments were performed using the TNT reticulocyte lysate system (Promega). Atrial and ventricular cell plasma membranes were isolated from bovine heart¹². To visualize proteins immunoprecipitated



from heart, plasma membrane proteins were first biotinylated by NHS-LC-biotin (Pierce)¹³. Biotinylated proteins were solubilized in solution containing (in mM): 10 Na-HEPES (pH 7.5), 300 NaCl, 2 dithiothreitol, protease inhibitors (2 μ g ml⁻¹ aprotinin, leupeptin and pepstatin, 0.2 mM phenylmethylsulphonyl fluoride) and 1% Triton X-100. Solubilized proteins were immunoprecipitated by 5 μ g antibody per mg of solubilized protein, or per 25 μ l of *in vitro* translation reaction, and 10 μ l of protein A-Sepharose Fast Flow (Pharmacia) for 2 h at 4 $^{\circ}$ C, followed by wash in the same buffer. Immunoprecipitated biotinylated proteins were visualized by streptavidin-horse radish peroxidase conjugate (Pierce) and enhanced chemoluminescence reagents (ECL; Amersham). Membrane proteins were deglycosylated with N-glycosidase F (Boehringer). For expression of full-length GIRK1, the 5' UTR and coding sequence of GIRK1⁴ was subcloned into the pBluebacII baculovirus transfer vector (Invitrogen). Sf9 cells were infected at a multiplicity of infection (MOI) < 1 for 110 h. *In vitro* transcribed GIRK1 mRNA (8 ng) was injected into *Xenopus* oocytes defolliculated by collagenase treatment. Solubilized membrane proteins from 10 oocytes incubated for 72 h after injection were western-blotted using aCsh.

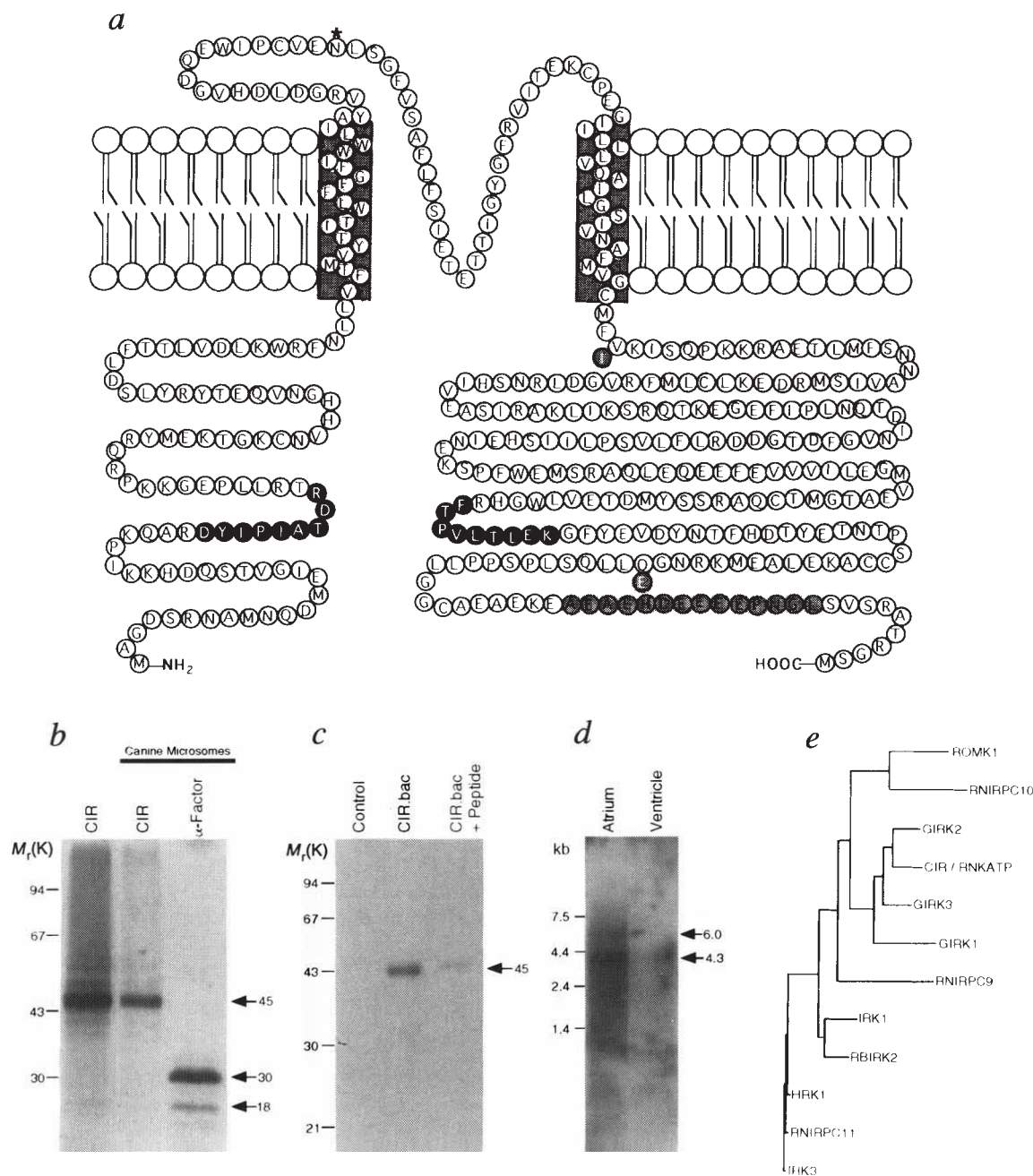


FIG. 2 p45 is CIR. *a*, Amino-acid sequence of CIR. Darkened regions (amino acids 30–40 and 333–342) indicate the peptide fragments sequenced after immunoaffinity purification of p45. Shaded residues 396–409 represent antigenic peptide used to generate an anti-CIR antibody used in *c*. The alternative residues at position 188 (isoleucine for valine) and position 375 (glutamine for glutamate) denote differences between CIR sequence and RNKATP (ref. 7). The consensus glycosylation sequence is denoted by an asterisk. *b*, *In vitro* translation of the CIR cDNA produced a single protein band with an estimated M_r of 45K. CIR is not glycosylated in the presence of canine microsomes. *Saccharomyces cerevisiae* α -factor served as a positive control for effective glycosylation (30K band represents the glycosylated product of the non-glycosylated 18K N factor). *c*, An affinity-purified anti-peptide (amino acids 396–409; shaded segment in *a*) antibody was used to probe a western blot of Sf9 cells. Sf9 cells infected with CIR.bac produced a single protein product of M_r 45K. Immunostaining was almost completely competed off with a 1,000-fold molar excess of antigenic peptide. *d*, Northern blot analysis of rat heart showing greater abundance of CIR mRNA in rat atria. *e*, Phylogenetic tree of representative members of inwardly rectifying K^+ channels. The tree was generated

using the Kimura protein distance analysis and neighbour joining algorithm (GCG, Univ. Wisconsin).

METHODS. p45 was immunoaffinity-purified on immobilized aCsh (CNBr-Sepharose; Sigma) from 100 mg of solubilized bovine atrial plasma membrane proteins in conditions analogous to those used for immuno-precipitation. Bound proteins were eluted by a CHAPS-containing buffer (pH 2.3) and separated by SDS-PAGE. The band corresponding to p45 was excised from the gel, digested by trypsin and CNBr¹⁴ and micro-sequenced. *In vitro* translation of CIR was as described for Fig. 1. CIR.bac was produced by subcloning the coding sequence of the CIR cDNA into pBlueBac III (Invitrogen). Sf9 cells were infected at a MOI of 5 PFU per cell. After 3 days, the cells were isolated and solubilized in SDS under reducing conditions; 2 μ g of protein from infected and uninfected Sf9 cells was subjected to SDS-PAGE, followed by western blotting using affinity-purified anti-CIR antibodies. The anti-CIR antibody was raised against a synthetic peptide corresponding to amino acids 396–409 conjugated to KLH. For northern analysis, a blot containing rat atrium and ventricle mRNA (3 μ g each) was probed with an entire rat CIR cDNA probe. This blot was hybridized and washed under high stringency conditions (final wash $0.1 \times$ SSC at 65 °C).

FIG. 3 Heterologously expressed CIR is activated by G proteins. *a*, Application of 5 μ M acetylcholine to oocytes injected with m2 and CIR mRNAs (right, $n=3$) elicits a small Ba^{2+} -sensitive (200 μ M) inward current not observed in m2-injected oocytes (left, $n=3$). Oocytes were voltage-clamped at -70 mV. 'High K^+ ' refers to the switch from 2 mM K^+ to 96 mM K^+ . The dashed line indicates the zero current level. *b*, 'Spiky' channels observed in cell-attached patches from Sf9 cells infected with CIR.bac ran down completely upon excision in an ATP- and GTP- γ S-free bath. Addition of 200 μ M GTP- γ S reactivated the channels. Spaces in the data trace represent time (10–20 s) in which the patch was excised and GTP- γ S was added to the bath. *c*, CIR activation by ATP and GTP- γ S in CHO cells transfected with CIR. In the top trace, cell-attached channel activity disappeared on formation of the inside-out patch. Addition of 10 μ M GTP- γ S resulted in no immediate stimulation, but subsequent addition of 100 μ M ATP (pH 7.2) resulted in a rapid increase in inwardly rectifying channel activity. In the lower trace, addition of 100 μ M ATP to a quiet inside-out patch resulted in the slow development of channel activity. Subsequent addition of 10 μ M GTP- γ S resulted in a rapid increase in channel activity. AMP-PNP (0.1–1 mM; $n=3$) did not support the GTP- γ S-mediated activation of the CIR channels. Voltage pulses to $+80$ mV reveal the strong inward rectification of these channels.

METHODS. Stage V and VI *Xenopus* oocytes were defolliculated by collagenase treatment and injected with 8 ng of CIR mRNA and 1 ng of m2 receptor mRNA. mRNAs were transcribed *in vitro* from cDNA clones (mCAP; Stratagene). After 72 h, currents were recorded using two-electrode voltage clamp (NPI Turbotec; Darmstadt), filtered at 1 kHz (8-pole Bessel filter). The bath solution contained (in mM): 96 KCl, 2 NaCl, 1.8 CaCl_2 , 1 MgCl_2 and 5 Na-HEPES (pH 7.4). Cells infected by CIR.bac at a MOI of ~ 100 plaque-forming units (PFU) per cell were assayed 24–120 h post-infection. The cDNA encoding murine CD4 (L3T4; supplied by D. Littman, UCSF) was subcloned into the cytomegalovirus-based (CMV) mammalian expression vector pRBG4 (supplied by S. Sine, Mayo Foundation). The CIR-coding region was subcloned into pCDNA1/Amp (Invitrogen) downstream from CMV promoter. CHO (and HEK-293) cells plated on 10-cm Petri dishes at 50% confluency were transfected by calcium phosphate precipitation¹⁵ by CIR (10 μ g) and 5 μ g of the L3T4 construct. At 24 h post-transfection, the cells were incubated at 4 °C for 10–15 min with 5 ml of phycoerythrin-conjugated anti-mouse CD4 monoclonal antibodies (Pharmingen; 0.4 mg ml^{-1}) and washed with PBS. Fluorescently labelled CD4-expressing CHO cells were assayed for novel currents. The pipette and bath solutions were identical: 140 mM K^+ ,

144 mM Cl^- , 10 mM HEPES, 5 mM EGTA and 2 mM Mg^{2+} (pH 7.2). Currents were recorded using an Axopatch 200A patch clamp amplifiers, filtered at 5 kHz (8-pole Bessel filter) and digitized at 50 kHz (PCLAMP6).

current. Despite the short-lived channel openings, CIR currents in all three mammalian expression systems demonstrated distinct properties of ion channels. The current showed strong Mg^{2+} -dependent inward rectification, K^+ -selectivity, block by 100 μ M external Ba^{2+} (data not shown) and regulation.

GIRK1/CIR functional coexpression

***Xenopus* oocytes.** Given the compelling evidence that GIRK1 and CIR proteins physically associate in heart (Fig. 1), we coexpressed GIRK1 and CIR in several cell systems. When either GIRK1 or CIR were individually expressed with m2 receptor in *Xenopus* oocytes, acetylcholine elicited modest inwardly rectifying K^+ currents. The average peak current measured at -80 mV in 96 mM $[\text{K}^+]_o$ was -0.39 ± 0.05 μ A ($n=48$) for CIR and -0.90 ± 0.16 μ A ($n=32$) for GIRK1 alone, compared with -0.32 ± 0.03 μ A ($n=31$) in control oocytes. However, when CIR and GIRK1 were expressed together (with m2 receptor), a much larger basal (agonist-independent) and ACh-induced inwardly

rectifying K^+ current was routinely measured (Fig. 4). For CIR/GIRK1 coexpression, ACh-induced inward current averaged $-7.15 \mu\text{A} \pm 0.64$ ($n=31$) at -80 mV, eight times the average current measured with GIRK1 alone and 18 times that in CIR-expressing oocytes. Western blots of membranes from oocytes expressing GIRK1 alone or coexpressing GIRK1 and CIR indicated that the large increase in current is apparently not due to an increase in GIRK1 protein expression (data not shown). ACh-induced inward current resulting from GIRK1/CIR/m2 coexpression was blocked by Ba^{2+} (effective concentration for half-maximum response (EC_{50}) = 0.5 mM) and Cs^+ (EC_{50} = 0.1 mM). GIRK1/CIR current was K^+ -selective as determined by plotting current-reversal potentials as a function of $[\text{K}^+]_o$ (slope = -53 mV per decade).

Previous cell-attached patch recordings from GIRK1-expressing oocytes revealed that they had a higher basal channel activity³ than atrial cells¹. Also, the regulation of atrial I_{KACH} by GTP- γ S and $\text{G}_{\beta\gamma}$ is apparently more robust than in oocytes

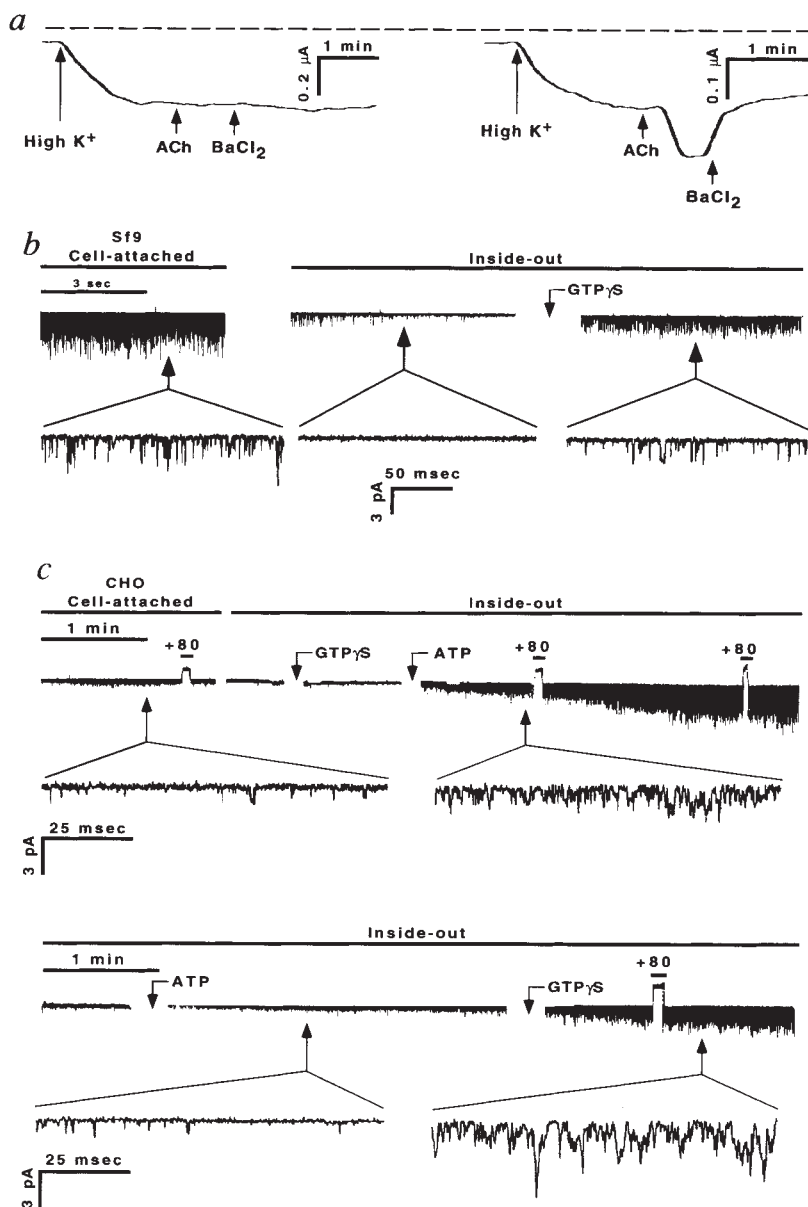
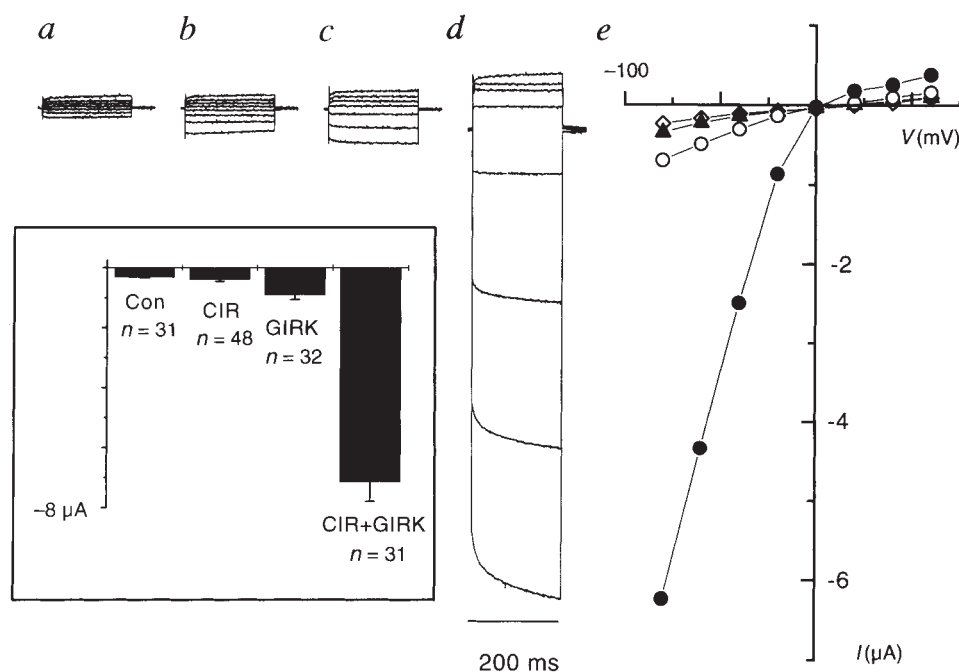


FIG. 4 Coexpression of GIRK1 and CIR in *Xenopus* oocytes dramatically increased acetylcholine-stimulated inwardly rectifying currents. *a–d*, Current traces from oocytes injected with 50 nI H₂O (*a*) CIR/m2 (*b*) GIRK1/m2 (*c*) and CIR/GIRK1/m2 (*d*). GIRK1 and CIR mRNA was injected at 8 ng each per oocyte with 1 ng per oocyte m2 mRNA. Currents were recorded 5–15 s after 5 μ M ACh bath application. Holding potential was -10 mV. Voltage pulses were applied from -80 to 60 mV in 20 -mV steps. *e*, Current-voltage relation for data in *a–d*. Currents taken 180 ms after the beginning of the pulse were plotted. Symbols represent water ($\diamond$), CIR and m2 ($\blacktriangle$), GIRK1 and m2 ($\circ$), and CIR, GIRK1 and m2 ($\bullet$). Current scale in *e* applies to *a–d*. *f*, Average peak currents at -80 mV. Oocytes were measured 2 days after mRNA injection.



expressing GIRK1 channels^{2,3,8}. We attempted to compare these parameters in oocytes expressing GIRK1, CIR or GIRK1/CIR. In general, our data obtained with GIRK1 expression in oocytes are comparable to those of previous reports^{3,8}. However, the low level of channel expression and the presence of an abundant stretch-activated channel hampered routine measurement of GIRK1 single channels. Similarly, novel single channels were not detected in oocytes after CIR expression in 12 cell-attached patches or in excised patches in the presence or absence of $G_{\beta\gamma}$ ($n=5$) or GTP- γ S ($n=4$). In contrast, when GIRK1 and CIR

were coexpressed, I_{KACH} -like channels were readily observed (36/41 patches; Fig. 5). The GIRK1/CIR single-channel conductance was ~ 35 pS in symmetrical 140 mM $[K^+]$ and the mean open-time histogram was fitted by a single exponential with $\tau=1.3$ ms (Fig. 5). We routinely observed 10–200-fold stimulation of channel activity with 200 μ M GTP- γ S or 50 – 100 nM $G_{\beta\gamma}$, and the single channels resulting from GIRK1/CIR coexpression were not substantially different from those resulting from GIRK1 expression alone. From these results, we conclude that the primary effect of coexpression of GIRK1

FIG. 5 GIRK1/CIR single-channel properties in oocytes. *a*, At least three channels were activated by 200 μ M GTP- γ S applied to the intracellular side of the patch. *b*, Time course of CIR/GIRK1 activation by GTP- γ S in an inside-out patch plotted as the integral of channel activity (NP_o , where N =number of channels and P_o =probability of channel opening). *c*, Open-time histogram of GIRK1/CIR channels activated by GTP- γ S and measured at -80 mV. The open times were fitted to a single exponential ($\tau_{open}=1.3$ ms). *d*, Open channel I - V relation measured by applying a voltage ramp from -100 to 100 mV on a rare long-lived burst of channel openings (36 ms ramp duration). The pipette and bath solutions contained (in mM): $134K^+$, $138Cl^-$, $2Mg^{2+}$, 10 HEPES, pH 7.2 . $GdCl_3$ (10 μ M) was added to the pipette solution to inhibit abundant stretch-activated channels. For mean open-time and conductance determinations, the filter cutoff was 5 kHz.

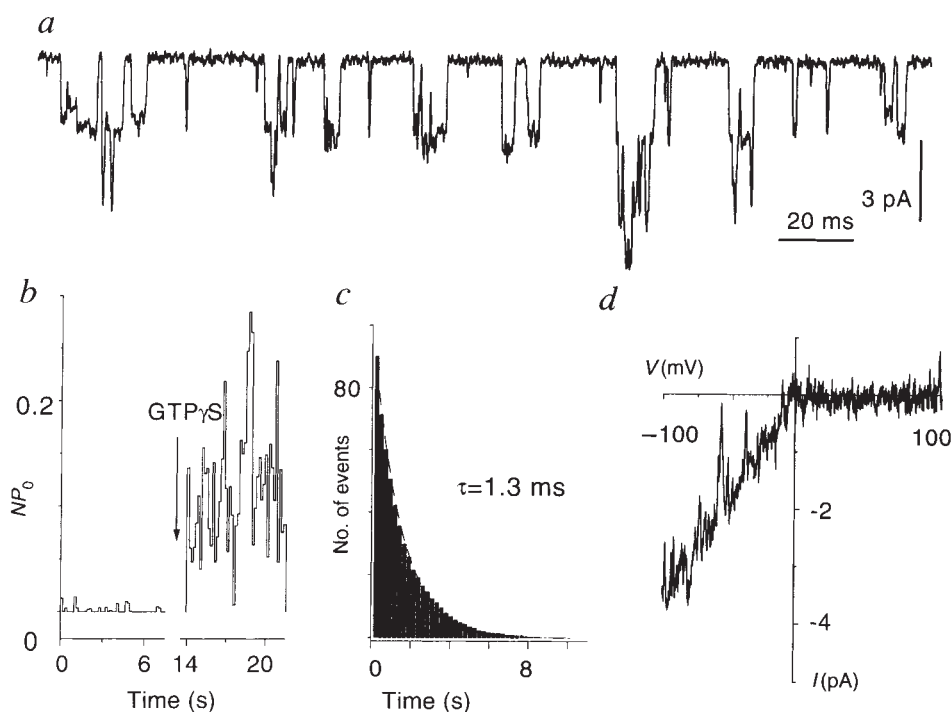


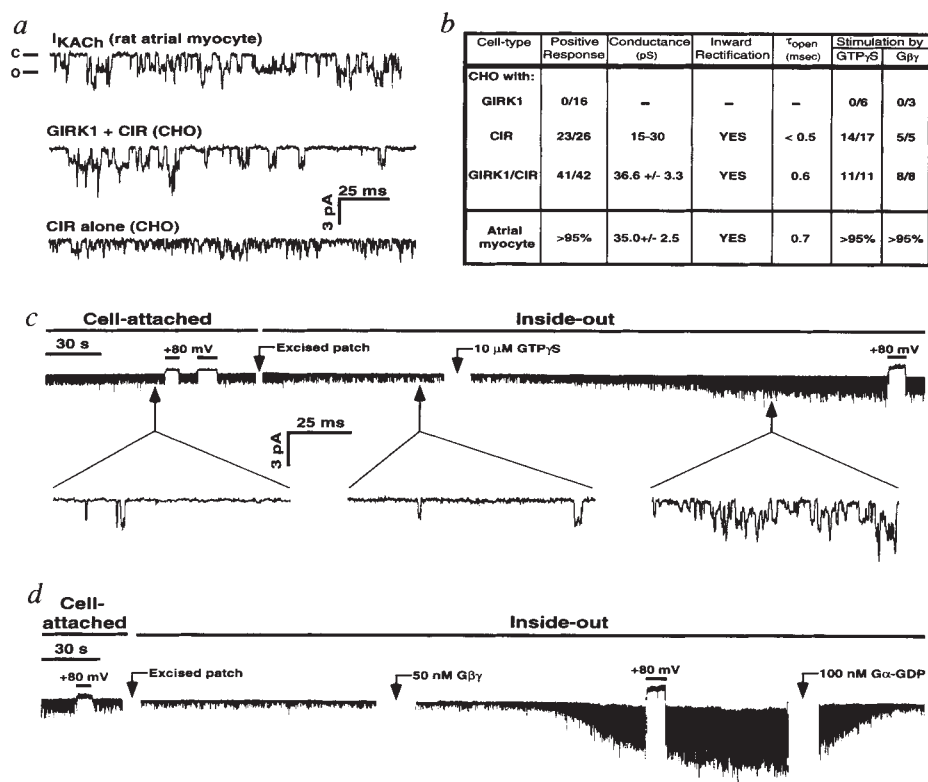
FIG. 6 Channels produced by GIRK1 and CIR coexpression in CHO cells are similar to atrial I_{KACH} channels. **a**, Comparison of inside-out patch recordings from rat atrial myocytes, CHO cells expressing GIRK1/CIR, and CIR alone, after stimulation by GTP- γ S ($V_p = +80$ mV); o, open; c, closed level. No novel channel activity (compared with control cells) was observed in cells transfected with GIRK1 alone ($n = 16$). **b**, Summary of the characteristics of the channels observed in transfected CHO cells, and comparison with native I_{KACH} . 'Positive response' indicates the number of cells out of the total number tested that demonstrate the corresponding novel channel activities. **c**, Representative experiment from a CHO cell coexpressing GIRK1 and CIR, showing 200-fold stimulation by GTP- γ S. Pulses to +80 mV show the strong inward rectification of these channels. Patch excision resulted in a 3–10-fold reduction in channel activity. **d**, Stimulation of GIRK1/CIR channel activity in a CHO cell patch by 50 nM bovine brain $G_{\beta\gamma}$ and reversal by 100 nM bovine brain G_{α} -GDP.

METHODS. The 5'-UTR and coding region of GIRK1 (KGA obtained from H. Lester) was subcloned into pRBG4 (see Fig. 3 for methods). CHO cells were transfected with GIRK1 (10 μ g), CIR (10 μ g) or GIRK1 + CIR (10 μ g each) according to Fig. 3. Bovine brain $G_{\beta\gamma}$ was prepared as described². Pipette and bath solutions were as described for Fig. 3. Conductance values for native I_{KACH} and GIRK1/CIR cotransfected CHO cells were obtained from the slope of the best linear fit of the mean amplitude values for >1,000 channel events at potentials ranging between -100 mV and -30 mV. Mean open-time values were obtained from

and CIR is to increase the number of functional I_{KACH} -like channels.

Mammalian cells. Cell-attached channel activity in CHO cells transfected with GIRK1 alone ($n = 16$) did not differ from that of wild-type or mock-transfected cells ($n = 10$). Patch excision (inside-out) followed by incubation with $G_{\beta\gamma}$, GTP- γ S and ATP ($n = 8$) did not elicit novel channel activity in transfected cells. Expression of CIR alone gave reproducible cell-attached channel activity (26/29, Fig. 6a, b), with the properties already described (Fig. 3c). Cotransfection of CHO cells with GIRK1 and CIR gave highly reproducible (41/42) cell-attached channel activities with characteristics similar to those of atrial I_{KACH} . These channels exhibited Mg^{2+} -dependent inward rectification and rundown (3–10-fold) upon patch excision, and were stimulated strongly by 20–50 nM $G_{\beta\gamma}$ or 10 μ M GTP- γ S (10–200-fold; Fig. 6c). ATP was not required for, nor did it seem to enhance or modulate significantly, the effects of GTP- γ S and $G_{\beta\gamma}$. Stimulation by both GTP- γ S and $G_{\beta\gamma}$ could be reversed by a two- to fourfold molar excess of G_{α} -GDP (3/3; Fig. 6d), suggesting that $G_{\beta\gamma}$, and not activated G_{α} , was responsible for the observed activity. Comparison of mean open time and current-voltage relationships for channels observed in cotransfected CHO cells with I_{KACH} from atrial myocytes attests to the similarities between these channels. Figure 6b summarizes the characteristics of the channels observed in CHO cells expressing GIRK1, CIR and GIRK1/CIR.

By size-exclusion chromatography, the atrial GIRK1/CIR complex eluted at 480–520K. Size-exclusion chromatography of membrane proteins from Sf9 cells infected by recombinant CIR/GIRK1 baculoviruses also indicated the formation of a large



inside-out patches held at -80 mV. Data were filtered at 5 kHz, with open-time histograms truncated to remove data binned at times <0.2 ms. Histograms were fitted adequately by a single exponential. NP_o values were determined as described previously for 5–10-s stretches of recording².

hetero-oligomeric complex containing both GIRK1 and CIR proteins (not shown). If no additional proteins are part of the complex, these data indicate that the atrial GIRK1/CIR complex is comprised of more than four subunits. However, definitive determination of native GIRK1/CIR subunit stoichiometry awaits further study.

Discussion

Many ligand-, voltage- and second-messenger-gated channels are heteromultimers. With the recent cloning of members of the inwardly rectifying K^+ -channel family, it became feasible to examine the native structures of this important family of ion channels. One of the most well studied inward rectifiers, I_{KACH} , has been the focus of considerable research due to its regulation by G proteins. Using antibodies specific for regions of a protein thought to encode I_{KACH} , we examined the structure of native I_{KACH} . We demonstrate here that native I_{KACH} is comprised of two homologous subunits, products of the GIRK1 and CIR genes. As GIRK1⁸ and CIR (this report) subunits seem to be regulated by $G_{\beta\gamma}$, both subunits may contribute to the known $G_{\beta\gamma}$ regulation of I_{KACH} . Clearly, the elucidation of the domain within I_{KACH} responsible for regulation by G proteins will require direct examination of the heteromultimer. To this end, our preliminary binding studies show that the atrial GIRK1/CIR complex, Sf9-derived CIR and Sf9-derived GIRK1 all interact with $G_{\beta\gamma}$. The specificity of these interactions is under investigation.

How is it that GIRK1 was expression-cloned without the addition of CIR? An obvious explanation is that GIRK1 forms functional homomultimeric channels with characteristics similar to those of I_{KACH} . A potentially more interesting explanation is

that a CIR homologue is endogenously expressed in oocytes. Indeed, preliminary low stringency northern analysis indicates that oocytes possess a CIR homologue. This hypothesis is further supported by our expression studies in mammalian cells; we have been unable to demonstrate channels resulting from GIRK1 expression in CHO or HEK cells, despite the expression of GIRK1 protein (not shown).

CIR is identical in all but two amino acids to a protein recently reported to form an ATP-sensitive K^+ channel (Genbank accession number X83584; see also ref. 7). However, the current measured in CIR-expressing oocytes, Sf9 cells, HEK and CHO cells, is novel and, to our knowledge, has not been observed in native cells or tissues. The ion-channel properties of CIR alone expressed in four cell systems are unlike those of I_{KATP} in kinetics, conductance or rectification. The expressed channels were not inhibited by intracellular ATP or opened by pinacidil. Furthermore, the prevalence of CIR mRNA in atria contrasts with the more predominant ventricular distribution of functional I_{KATP} (ref. 9). It is reasonable to assume that CIR is present in heart only as a subunit of I_{KACH} and does not form homomultimers, or pure CIR channels, in nature. If this is so, the

properties of CIR channels we have observed may be manifested only in artificial systems in which high levels of protein are expressed and a defective ion channel is formed. An alternative hypothesis is that CIR forms a functional channel possessing multiple sub-states and frequently blocked openings that are too fast to resolve. A more interesting possibility is that CIR combines not only with GIRK1 to form I_{KACH} , but with different channel subunits to yield other highly regulated channels such as I_{KATP} .

There are two main conclusions from our work. First, we consider that the functional I_{KATP} channel protein complex remains unidentified. We speculate that I_{KATP} may be formed by heteromultimeric combinations of inward rectifier subunits, perhaps including the sulphonylurea receptor¹⁰ as a regulator. More importantly, we have shown for the first time the heteromultimeric nature of an inwardly rectifying K^+ channel. The heteromultimeric channels differ in function from homomultimers. Inward-rectifier K^+ -channel clones should thus be classified by homology rather than function. This finding predicts a wealth of possible channels comprised of different subunit combinations differentially regulated by signalling systems. □

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LETTERS TO NATURE

Relativistic motion in a nearby bright X-ray source

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THE recent discovery¹ of radio components apparently moving away from a Galactic source of transient X-ray emission faster than the speed of light (superluminal motion) has identified a low-energy Galactic counterpart to quasars. Here we report high-resolution radio observations of a second Galactic superluminal radio source GRO J1655–40, which was detected as an X-ray transient² on 27 July 1994. Our radio images reveal two components moving away from each other at an angular speed of $65 \pm 5 \text{ mas d}^{-1}$, corresponding to superluminal motion at the

estimated distance of 3–5 kpc. The 12-day delay between the X-ray and radio outbursts suggests that the ejection of material at relativistic speeds occurs during a stable phase of accretion onto a black hole, which follows an unstable phase with a high accretion rate.

Our very-long-baseline interferometry (VLBI) images (Fig. 1) were made from data obtained with the SHEVE (Southern Hemisphere VLBI Experiment) array^{4,5}. The observations span the four days 21–24 August 1994, beginning roughly nine days after the onset of the large radio outburst. They reveal a linear, highly collimated structure along position angle 48° , dominated by two main components separated by $\sim 0.5 \text{ arcsec}$, each of which shows internal structure. The northeast component can be modelled by three subcomponents on the first two days and two subcomponents on the final two days. The southwest component shows a compact, bright but rapidly fading subcomponent, with an extension towards the northeast. The resemblance to the rapidly moving and evolving 'jets' containing bright subcomponents ('knots') familiar in distant quasars and active galactic nuclei is striking. From our identification of the northeast subcomponents between the images we estimate that they move with the same speed, $65 \pm 5 \text{ mas d}^{-1}$, relative to the compact subcomponent in the southwest. For comparison, the subcomponents of GRS1915+105 were found to have proper motions of 17.53 and 9.04 mas d^{-1} , and SS433 8.77 mas d^{-1} (ref. 6).

The extremely high angular motion observed in GRO J1655–40 violates the fundamental assumption of synthesis imaging that the source structure should remain constant throughout each observation. In fact the source expanded by an angle comparable to the size of the synthesized beamwidth in the course of each 12-hour observation. As no general imaging

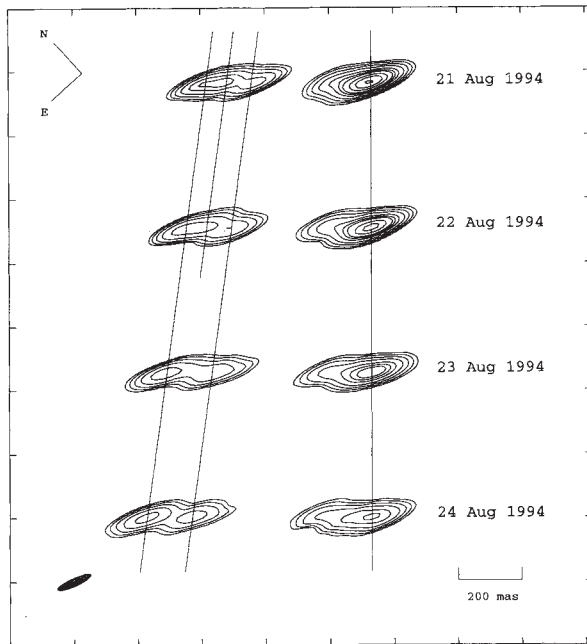


FIG. 1 The four VLBI images at 2.29 GHz. Data were recorded in right circular polarization with the 2-MHz bandwidth of the MKII system, and correlated at the Caltech/JPL Block II processor. The contour levels are 1, 2, 4, 8, 16, 24, 32, 64, 80 and 95 per cent of the peak flux density in the montage of 0.59 Jy per beam. The dynamic range is 100:1. The images have $1,024 \times 1,024$ pixels, each 2 mas across, and have each been rotated anti-clockwise by 42° and aligned to register the southwest component vertically. The restoring beam is $123 \text{ mas} \times 26 \text{ mas}$ with major axis position angle of 72° . The observations included Australian antennae at Tidbinbilla, Parkes, Hobart, Culgoora and Mopra. Antennae were also used in the USA at Goldstone and Mauna Kea, and in South Africa at Hartebeesthoek but the source was not detected on any inter-continental baseline.

algorithms yet exist for this situation, we made extensive simulations to assess the effects of the rapid motion on our images and to develop appropriate strategies. We created and imaged artificial data from a hypothetical double radio source whose separation of ~ 0.5 arcsec increased linearly during the observation. We explored the effects of several different expansion rates.

From the simulations we found that the images produced in the normal manner from each 12-hour observation did contain subtle but significant distortions due to the source motion, although these were not as serious as might be expected. In particular, a gentle but noticeable curvature in the overall morphology of the source, with the components distributed along an arc rather than a straight line, was shown to be an artefact. The simulations did confirm, however, that the derived rate of angular expansion, with each individual image deemed to represent the source at a time corresponding to the observation midpoint, was a robust parameter that was never more than 5% in error over a broad range of initial conditions. Further simulations showed that images made from a single 5-hour section of a complete 12-hour observation were free of discernible distortions due to source motion, although the sensitivity and overall image quality were necessarily reduced.

We re-imaged our data accordingly, using the first 5 hours of data from each 12-hour observation to produce the images in Fig. 1. This initial period contains more structural information about the source than the latter 7 hours, including several full beats in visibility amplitude as well as the primary peak. It is also the period in which the signal-to-noise ratio tended to be higher on all baselines. The expansion of the source during the 5 hours was 13 mas, less than half of the small dimension of the synthesized beamwidth.

Optical spectra of GRO J1655-40 taken during the decline of the initial X-ray outburst⁷ confirm it as a Galactic object and indicate significant reddening due to absorption, despite its relatively high Galactic latitude (Galactic longitude and latitude $l = 345.0^\circ$, $b = +2.2^\circ$ respectively). We have estimated the distance to GRO J1655-40 by examining its spectrum near the H I transition of atomic hydrogen at 1,420 MHz. The spectrum in Fig. 2 shows absorption features over the range $+10 \text{ km s}^{-1}$ to -30 km s^{-1} , with a further weak feature at -50 km s^{-1} , confirmed with multiple observations. Comparison spectra taken in the same direction but with low angular resolution (0.5°) show H I emission extending out to -150 km s^{-1} . Attributing the absorption out to -30 km s^{-1} to H I clouds participating in general Galactic rotation places a lower limit of 3.0 kpc on the distance. The weak feature at -50 km s^{-1} , if due to Galactic rotation, implies a minimum distance of ~ 4.2 kpc, but it cannot be ruled out that this feature is due instead to H I driven by a 50 km s^{-1} expanding shell of ionized material surrounding the adjacent Scorpius OB1 association⁸, at a distance of 1.9 kpc.

It is difficult to set a categorical upper limit to the distance owing to the relatively high Galactic latitude of GRO J1655-40, and the consequently smaller numbers of H I clouds along the line of sight beyond ~ 5 kpc. But given the presence of detectable H I emission along adjacent lines of sight out to -150 km s^{-1} , it is unlikely that the distance is much greater than 5 kpc. For comparison, the Galactic H II region CTB35A in a similar direction ($G345.4+1.4$) has H I absorption out to -24 km s^{-1} and is thought to be at a distance of ~ 2.5 kpc (ref. 9). We therefore estimate the distance of GRO J1655-40 to be 3.0–5.0 kpc. The measured angular motion is therefore equivalent to an apparent speed relative to the speed of light of $\beta_{\text{app}} = 1.5 \pm 0.4$.

We offer two plausible explanations for the source morphology. One of the components could be the site of ejection with the other component moving away from it in a one-sided expansion. Alternatively, if the site of ejection ('core') lies somewhere between the two components that we observe, then both would be in motion away from a common origin and the expansion two-sided. Unfortunately, self-calibrated VLBI images, such as those presented here, have an unknown offset in each angular coordinate, rendering absolute astrometric registration impossible. As we cannot distinguish between these geometries with our data, we shall consider both. In the one-sided case, the minimum intrinsic speeds of material in the jet relative to the speed of light¹⁰ are 0.7 and 0.9 for distances of 3 kpc and 5 kpc respec-

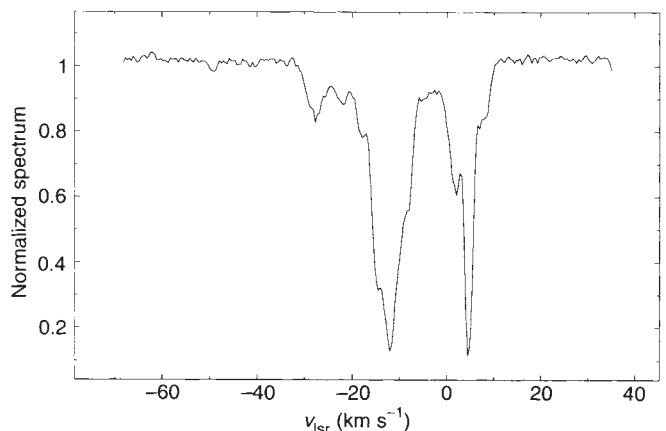


FIG. 2 The H I spectrum of GRO J1655-40 obtained from the Australia Telescope Compact Array at $6''$ resolution. Interpretation of the absorption features due to Galactic rotation and comparison of this profile with that of the nearby H II region CTB35A (ref. 9) give a probable distance of 3–5 kpc.

tively. The minimum velocity $\beta_{\min}$ in the two-sided case can be shown to be

$$\beta_{\min} = \begin{cases} \beta_{\text{app}}/2 & \text{if } \beta_{\text{app}} \leq \sqrt{2} \\ (\beta_{\text{app}}^2 - 1)^{1/2}/\beta_{\text{app}} & \text{if } \beta_{\text{app}} > \sqrt{2} \end{cases}$$

giving a corresponding range of values of 0.5 to 0.8. This expression assumes only equal and opposite velocities for the two components. Simultaneous ejection is not required and the core can lie anywhere between the two separating components. In either geometry, the intrinsic motion in the radio source is therefore at least mildly relativistic.

Extrapolating the outermost subcomponents backwards in time at the observed separation speed, we derive the zero separation time of August 13.5 ± 0.5 . This is roughly one day after the rapid rise in the radio flux density³ and close to the end of the decline of the X-ray luminosity¹¹. This is a most peculiar aspect of the behaviour of GRO J1655–40 and makes it unique among Galactic X-ray sources. The appearance of the radio outburst 12 days after the X-ray outburst, when the X-ray flux decreased strongly, suggests that the mechanism which formed the X-rays also inhibited the formation and ejection of the radio components. The accretion of material onto a compact object offers a plausible explanation if initially the accretion disk is geometrically thick and radiation-pressure supported, emitting X-rays at essentially the Eddington limit during a supercritical accretion event, then becomes thin and stable after the accretion rate decreases. Thus, while the accretion rate was high and the disk thick, the formation and ejection of radio components was disrupted, with at best only a weak, optically thick radio component present. Once the disk became thin and stabilized, the ejection events could occur by well known mechanisms such as Blandford–Payne¹². This is consistent with the observed X-ray flux during the outburst, which remained constant at roughly the level corresponding to the Eddington limit for an object of 1 solar mass at a distance of ~ 4 kpc.

The identification of moving radio components in GRO J1655–40, along with those in GRS1915+105, SS433 and Cyg X–3 (ref. 13), indicates that such behaviour may not be uncommon in Galactic X-ray objects. Furthermore, it seems that a continuum of intrinsic speeds, currently known to range from $0.25c$ in SS433 to $0.92c$ in GRS1915+105, may characterize this new class of source. The link between relativistic motion and the possible signature of accretion, the delay of the emergence of the radio components until the decline of the X-ray luminosity in GRO J1655–40, may be an important clue to the nature of radio component production, not only in these objects, but also in the much more luminous and massive objects in active galactic nuclei. The detection of superluminal motion and the familiar jet or knot morphology in Galactic sources is itself important, and suggests an underlying unity of mechanism over an enormous range in scale, from the galaxy-scale outbursts seen in quasars and active galactic nuclei, to their comparatively feeble and smaller analogue seen here in GRO J1655–40. $\square$

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Antibody catalysis of a reaction otherwise strongly disfavoured in water

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SEVERAL examples have been reported recently¹ of antibody catalysis² of reactions that are strongly disfavoured because of the high free energy of the transition state. Here we show that catalytic antibodies can be used to promote a particularly useful kind of reaction from a synthetic point of view: one involving an intermediate that is highly unstable in water. We show that an antibody elicited against the quaternary ammonium ion 4a (Fig. 1) catalyses the protonation of the enol ether 1 to form, with complete enantioselectivity, an oxocarbenium intermediate. This species is highly reactive in water, and would normally react with a water molecule to give the corresponding ketone 2. But the antibody provides a hydrophobic environment that allows the oxocarbenium ion instead to undergo an intramolecular reaction to form an enantiomerically pure ketal 3. This result shows that catalytic antibodies can exclude solvent molecules entirely from crucial steps on the reaction pathway.

Oxocarbenium ion intermediates such as I (Fig. 1) and the glycosyl cation have lifetimes in water in the order of 10^{-5} – 10^{-12} s (refs 3, 4). In the acid-catalysed hydrolysis of enol ether 1, the rate-determining step with the transition state of highest energy is the formation of intermediate I (Fig. 1). Partitioning of I to yield products 2 and 3 occurs after the rate-determining step and involves lower energy barriers^{5,6}. Thus, for a catalyst to promote ketal formation, it should be able both to lower the energy barrier between enol ether 1 and intermediate I, and to lower the energy barrier for conversion of I to ketal 3 relative to ketone 2.

Monoclonal antibody 14D9, which binds hapten 4a with high affinity, catalyses several reactions involving oxocarbenium ion intermediates, including cleavage of a cyclic acetal⁷, hydrolysis of ketals⁸, and hydrolysis of enol ethers⁹. The latter reaction proceeds with high enantioselectivity of protonation at carbon and can be done on a preparative scale¹⁰. Catalysis occurs through stabilization of the intermediate oxocarbenium ion by an ionizable protein side chain⁹. A notable part of the binding energy between 14D9 and hapten 4a is provided by hydrophobic interactions with the methylpiperidinium moiety of the hapten, which implies that the antibody active site is highly hydrophobic¹¹. We therefore expected that this antibody might not only catalyse the formation of intermediate I by protonation of enol ether 1, but could also influence the partitioning step and lead to formation of ketal 3 by excluding water from the reaction centre.

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Acid-catalysed hydrolysis of enol ether 1 in aqueous solution gives ketone 2 as the sole product. No traces of ketal 3 can be detected, indicating that the substrate's hydroxyl group cannot compete against water on intermediate I. Antibody 14D9 catalyses the hydrolysis of 1. Catalysis is totally inhibited by hapten 4b and follows saturation kinetics. Although the catalytic reactions are done in 100% aqueous medium, about 12% of ketal 3 is found in the reaction products. Formation of products 2 and 3 follows Michaelis–Menten kinetics (Fig. 2), yielding comparable K_m values. This suggests that both the ketone and ketal products originate from a common intermediate that is formed catalytically in the antibody combining site.

The ratio between ketone 2 and ketal 3 formed in the antibody-catalysed reaction is constant throughout a broad pH

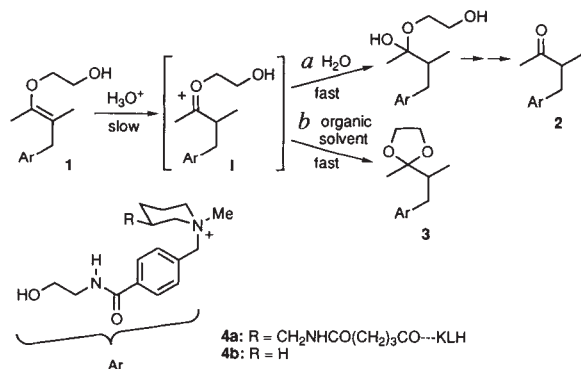


FIG. 1 Oxocarbenium ion intermediate I is generated by protonation of enol ether 1 under acid catalysis. In aqueous media, I is rapidly trapped by a molecule of water to yield a hemiketal and ultimately ketone 2 (pathway a). In organic solvents, under very low concentration of water, I can undergo ring closure to produce ketal 3 (pathway b). Although proton transfer is the rate-determining step in the overall conversion of 1 to either 2 or 3, the relative proportions of the two products is highly dependent on the medium. Monoclonal antibody 14D9, which binds hapten 4a with high affinity, catalyses the hydrolysis of 1 in 100% aqueous medium to produce a mixture of 2 and 3. Catalysis is totally inhibited by 4b and follows saturation kinetics.

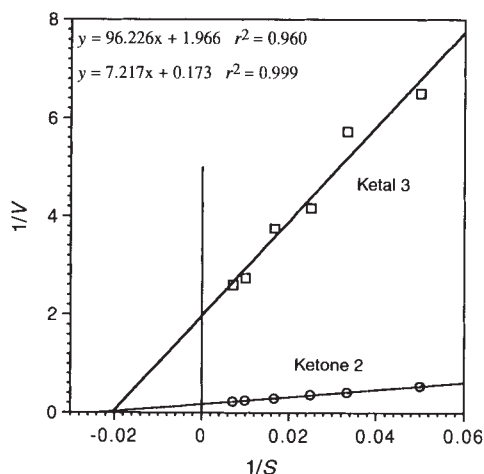


FIG. 2 A Lineweaver–Burk plot for the 14D9-catalysed hydrolysis of 1 to 2 and 3. General assay conditions: antibody (2–5 μM), substrate (20–150 μM), either PBS or 1,3-bis(tris(hydroxymethyl)-methylamino)propane buffered saline (50 mM buffer, 100 mM NaCl) at 25 $^{\circ}\text{C}$. All reactions were monitored at 254 nm by reverse-phase high-performance liquid chromatography (RP-HPLC; Hitachi L-6200A equipped with an AS-2000 autosampler and a Supelcosil LC-18 column (25 cm $\times$ 4.6 mm, 5 m) using 20:80 acetonitrile:water at 1 ml min $^{-1}$. All data shown here (rate, V , s $^{-1}$; concentration, S , μM) were obtained from reactions in PBS, pH 6.06, with the calculated K_m values for formation of compounds 2 and 3 being 42 and 50 μM , respectively. For the combined products: $K_{\text{cat}} = 1.2 \times 10^{-3}$, $K_{\text{uncat}} = 2.0 \times 10^{-6}$ s $^{-1}$.

range (between 5 and 9). Similarly, this product distribution is independent of the reaction temperature between 4 and 37 $^{\circ}\text{C}$. These observations suggest that the mechanistic pathways leading from intermediate I to either ketone or ketal are almost identical in terms of acid–base mechanism as well as thermodynamic activation parameters.

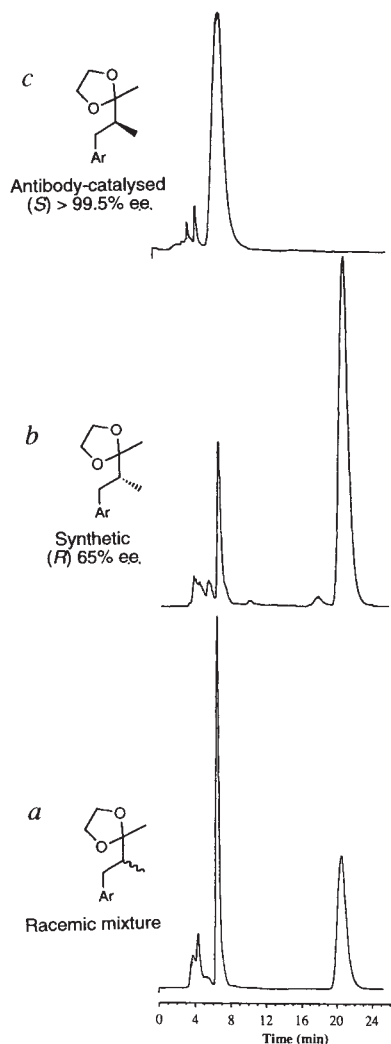


FIG. 3 Determination of absolute configuration and enantiomeric purity of ketal 3 produced in the antibody 14D9-catalysed reaction of 1. All analyses were done by HPLC (Perkin-Elmer 410 equipped with an ultra-violet detector, 254 nm) using a Chiracell OD-H column (Daicel Chemical Industries) with 20:80 isopropanol:hexane at a flow rate of 1 ml min $^{-1}$. a, HPLC chromatogram of a racemic mixture of 3. b, Chromatogram of (R)-3 that was synthesized from enantiomerically pure (1R, 2S)-(-)-norephedrine using the Evans methodology, proceeds with high diastereoselectivity and predictable absolute configuration¹⁵. The absolute configuration at the homobenzylic position was assigned on the basis of the many examples reported by Evans, including a closely related asymmetric alkylation reaction with unsubstituted benzyl bromide. c, Chromatogram of 3 that was produced by 14D9-catalysed reaction (note that a peak corresponding to the (R)-enantiomer cannot be detected even when the column is overloaded with a concentrated sample that causes peak broadening). As shown here, ketal 3 is obtained from the antibody-catalysed reaction with (S) configuration in >99.5% e.e. Similar HPLC analysis of ketone 2 isolated from the same antibody-catalysed reaction shows that it is formed with (S) configuration in 80% e.e. Considering that 20% of 2 is formed by the competing hydronium ion-catalysed hydrolysis of 1 under the reaction conditions, as determined by a control experiment, the observed enantiomeric purity of 2 (80% e.e.) implies that the reaction taking place in the antibody active site proceeds with greater than 97% enantioselectivity.

Both products 2 and 3 isolated from the same antibody-catalysed reaction are obtained with high enantiomeric purity and with the same (*S*) absolute configuration (Fig. 3). The remarkably high enantiomeric purity of ketal 3 (>99.5% enantiomeric excess, e.e.) agrees with the fact that 3 is formed exclusively by antibody catalysis. Furthermore, it also represents the first direct proof that the rate-determining protonation at carbon is achieved with complete enantioselectivity in the antibody active site. These observations confirm the assumption that both ketal 3 and ketone 2 originate from partitioning of a single intermediate and not from two diastereoisomeric transition states, which could be formed by protonation at either of the prochiral faces of the double bond in enol ether 1.

To assess the difficulty of what antibody catalysis has accomplished we have studied model systems. Formation of ketal 3 reflects low availability of water molecules at the vicinity of the intermediate I in the antibody active site. Such a medium effect is expected to exist within the hydrophobic active site of antibody 14D9^{9,11}. To test this hypothesis we used mixtures of either dioxane–water or isopropanol–water containing 5% acetic acid as a simple model environment¹². Acetic acid was chosen to mimic the residue responsible for general acid catalysis in the antibody active site. Measurable proportions of ketal are formed only at very low water concentration in acidic dioxane (Fig. 4a). The situation is even worse in acidic isopropanol, where less than 1% ketal could be detected at water concentrations greater than 1%. The product distribution of the antibody reaction corresponds to that observed in acidic dioxane containing only 3% water.

Very similar dependence of ketal proportions on water concentration is observed when perchloric acid (5×10^{-4} M) is used as the catalyst instead of acetic acid in either dioxane or isopropanol (both solutions, 5% acetic acid in water and 5×10^{-4} M perchloric acid in water, have pH 3.5), clearly showing that the partitioning is independent of the acid catalyst used. Similarly, reaction rates with HClO₄ decline by four orders of magnitude with decreased water concentration (from 100 to 5%). However, rates are increased steeply by one order of magnitude at lower concentration of water in both solvents (data not shown). This rate increase reflects enhanced reactivity of the hydronium ion as it becomes solvated by cosolvents which are less basic than water. This superacidity effect is not observed with acetic acid because acetate is more basic than water and therefore buffers the reactivity of the proton. Although the ionizable side chain responsible for catalysis in the antibody active site ($pK_a = 5.8$)^{9,13} should buffer the reactivity of the hydronium ion, a similar superacidity effect might explain part of the unusual rate enhancement observed with this catalyst.

Reaction rates drop by four orders of magnitude in these media as compared with pure water (Fig. 4b). By contrast, the antibody accelerates the reaction by three orders of magnitude. Attribution of ketal formation in the antibody active site to an effect of the medium would therefore lead to the conclusion that the antibody actually accelerates the reaction by seven orders of magnitude. Although the approximation of the antibody active site to a homogeneous, isotropic medium may be an oversimplification, this model is consistent with the observation of constant ketal/ketone ratios over broad ranges of pH values and temperatures.

As to the origin of ketal formation in the water–dioxane system, it is probably a dual effect of both lowered concentration of water molecules and increased alcohol nucleophilicity due to desolvation. Although the same factors could operate in the antibody active site as well, one should also consider the highly organized, asymmetrical environment within that site which may restrict the available conformations of intermediate I and thereby control its partitioning.

One of the greatest advantages of natural enzymes is their ability to handle reactive intermediates under ambient temperatures in the presence of water. For example, formation of a

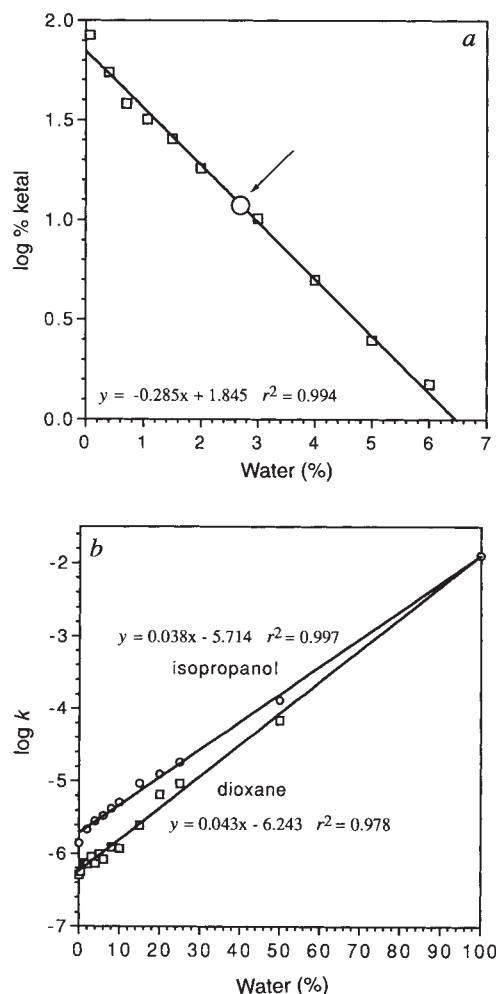


FIG. 4 Uncatalysed hydrolysis of 1 in organic solvents. *a*, Effect of water concentration on yields of ketal 3. All reactions were at 24 °C with 10^{-4} M substrate 1, using varying mixtures of dioxane and water and a constant concentration (5% v/v) of acetic acid. The circular data point represents the 12% ketal formed in the antibody-catalysed reaction. On this line it corresponds to 2.7% water in dioxane/acetic acid. *b*, Effect of water concentration on the reaction rate; log rate constants (s^{-1}) of the reaction either in isopropanol (circular data points) or in dioxane (rectangular points).

glycosidic bond by organic synthesis requires strictly anhydrous conditions in order to prevent hydrolysis of the highly reactive glycosyl cation. Glycosyl transferases, however, catalyse the same reactions in water¹⁴. We have shown here that it is possible to generate and handle water-incompatible, highly reactive intermediates within the active site of a catalytic antibody in aqueous solution. This has been demonstrated by the very difficult chemical task of ketal formation in water. The isolation of ketal 3 in very high enantiomeric purity is a striking example of the potential use of such chemistry. This proves that the 14D9-catalysed protonation at carbon is absolutely enantioselective. A wide array of synthetic transformations involving other water-incompatible, reactive intermediates should now be considered for antibody catalysis. □

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Increase in lower-stratospheric water vapour at a mid-latitude Northern Hemisphere site from 1981 to 1994

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WATER vapour in the atmosphere is the key trace gas controlling weather and climate, and plays a central role in atmospheric chemistry, influencing the heterogeneous chemical reactions that destroy stratospheric ozone. Although in the upper troposphere and lower stratosphere the radiative¹ and chemical² effects of water vapour are large, there are few measurements of water-vapour concentration^{3–10} and its long-term variation^{11–13} in this region. Here we present a set of water-vapour profiles for altitudes from 9 to 27 km, obtained at Boulder, Colorado, during 1981–94, which show a significant increase in water-vapour concentration in the lower stratosphere over this time. The increase is larger, at least below about 20–25 km, than might be expected from the stratospheric oxidation of increasing concentrations of atmospheric methane^{14,15}. The additional increase in water vapour may be linked to other climate variations, such as the observed global temperature rise in recent decades¹⁶.

In global climate models, nearly half of the projected increase in temperature due to a doubling of carbon dioxide in the atmosphere results from the effects of increased water vapour¹⁷. A doubling of water vapour in the stratosphere could lead to a 1 °C increase in surface temperature¹⁸. The present radiative forcing from the estimated rise in stratospheric water vapour resulting from methane oxidation is calculated¹⁶ to be about 10% of that for CO₂. But our limited knowledge of water vapour in the upper troposphere and lower stratosphere is one of the main uncertainties in climate modelling¹⁹.

Chemically, water vapour is the primary source of the hydroxyl radical in the atmosphere²⁰. Recent work^{21,22} has shown that water vapour concentration determines the effectiveness of the heterogeneous reactions that destroy ozone in the lower stratosphere. An increase in water vapour could thus lead to greater ozone loss.

The measurement of water vapour in the drier portions of the atmosphere, particularly above 5–10 km, is difficult^{3–6}. The inability of standard radiosondes to obtain such measurements and the generally high cost of suitable techniques has left a data gap in this region. Recently, satellite^{7,9} and aircraft measurements¹⁰ have shed new light on the variation of water vapour over a broader expanse of the globe, particularly in the stratosphere but also in the upper troposphere. Longer-term records of water vapour in this region are rare and limited to a few locations^{11–13}.

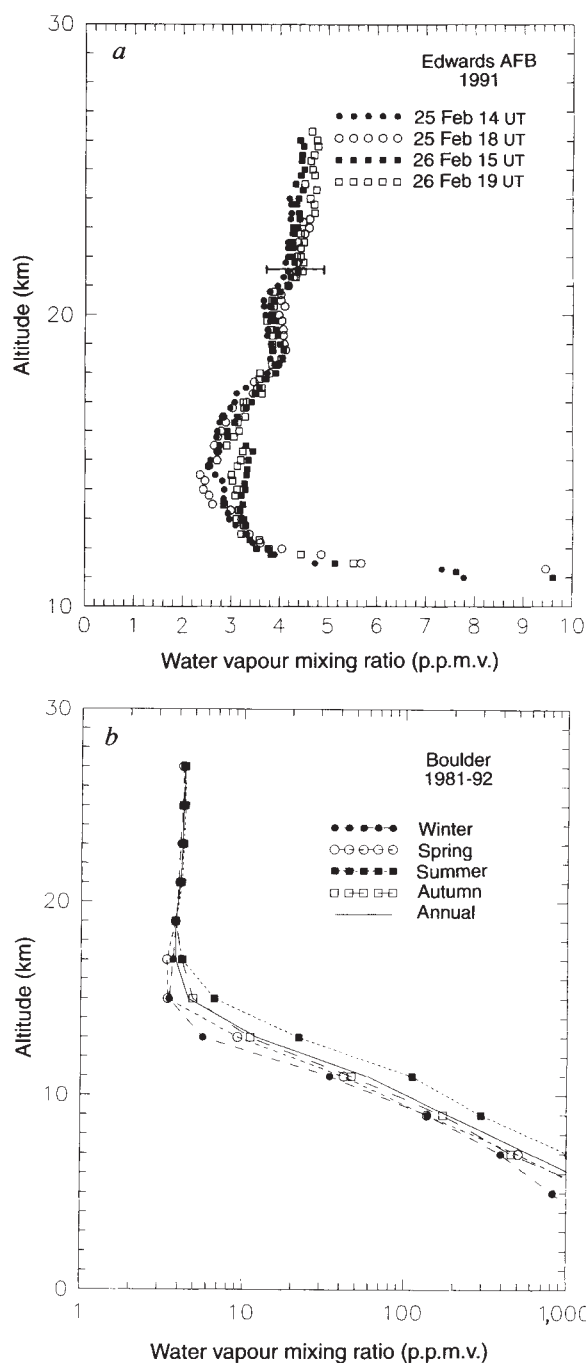


FIG. 1 Vertical profile mixing ratios for water vapour, in parts per million by volume (p.p.m.v.). a, At Edwards Air Force Base (AFB), California, for four soundings done within about a 30-h period in February 1991; b, for the seasonal and annual averages in the stratosphere at Boulder, Colorado, for the period 1981–94. In a, horizontal bar plotted at 21.5 km is $\pm 15\%$ of the mean and represents a conservative estimate of the instrument error. Above 18 km the symbol size in b represents one standard deviation about the mean.

The water-vapour profiles in this work were obtained using balloon-borne frost-point hygrometers. The instrument is a chilled-mirror design which uses a liquid cryogen, giving fairly fast response for an instrument of this type^{11,23}. The cryogen provides ample cooling to make measurements to altitudes of at least 30 km. The hygrometer operates on the principle that an equilibrium exists between the vapour pressure in the atmosphere and a water or ice surface only at a unique temperature (the dew or frost-point temperature). Using the Goff–Gratch²⁴ relationship, the mixing ratio of water vapour (relative to the

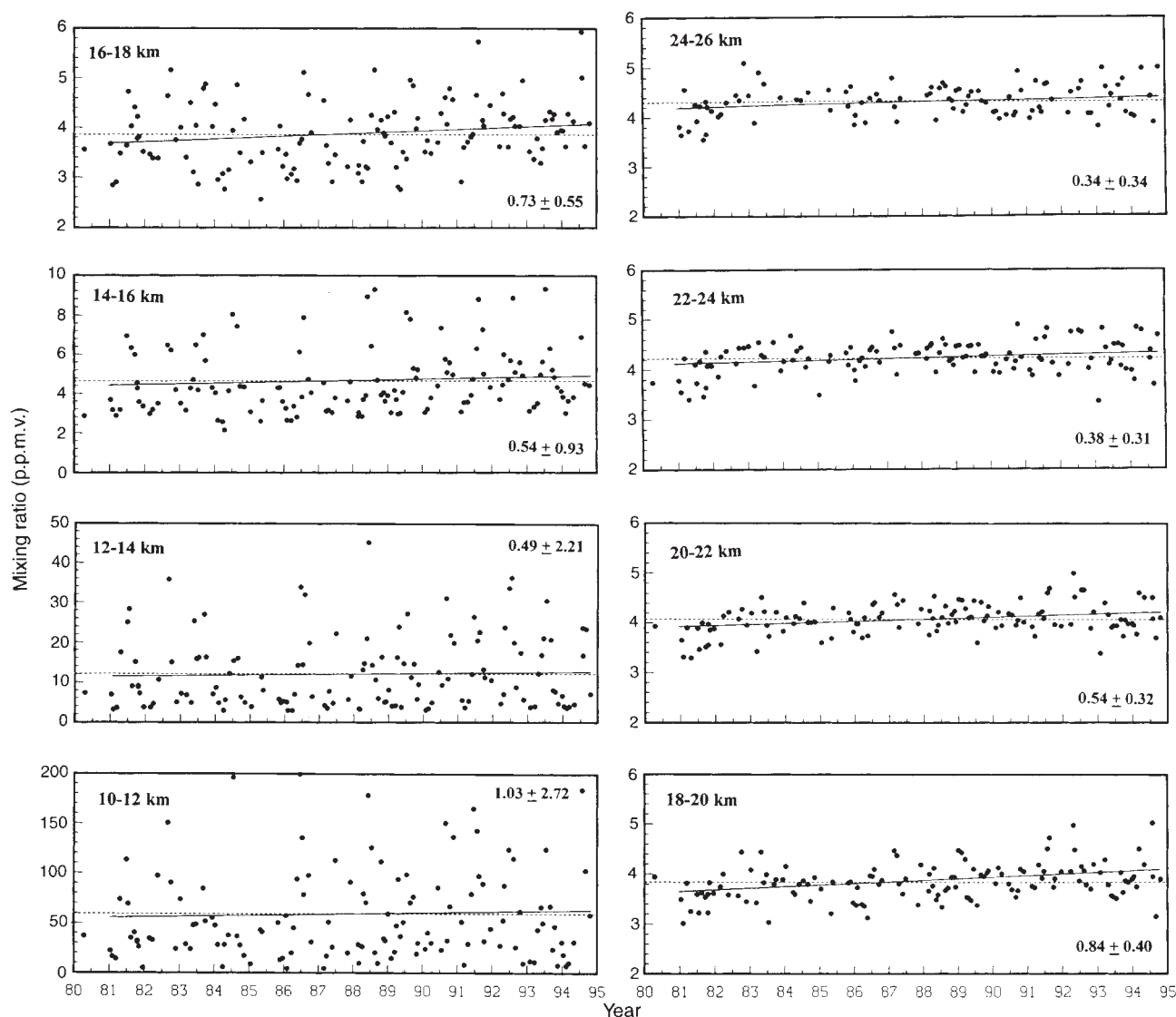


FIG. 2 Time series of water vapour mixing ratios in 2 km intervals for individual profile measurements at Boulder, Colorado, during the period 1981–94. The dashed line is the mean for each 2 km interval.

The solid line is the linear fit to the deseasonalized data. The number in each panel is the trend and 95% confidence interval in per cent per year.

vapour pressure over ice) is determined. Because the stratospheric mixing ratios are often several orders of magnitude smaller than those encountered near the surface (4–5 parts per million by volume (p.p.m.v.) rather than 10^4 p.p.m.v.), water vapour contamination from the instrument and balloon is the overriding concern in the stratosphere, especially at high altitude where low pressures result in efficient balloon out-gassing. To overcome this problem, we use only data obtained on controlled balloon descent for altitudes above 15 km. Optimization of the instrument to measure under dry conditions and balloon operational considerations limit the useful altitude range of the measurements to between about 8–28 km.

Each profile is obtained using a different instrument. The bead thermistor mounted in the face of the chilled mirror to measure the frost-point temperature is individually calibrated using a temperature standard traceable to the United States National Institute of Standards and Technology. The repeatability of the results is illustrated in Fig. 1a, which shows the stratospheric portion of four profiles obtained within a two-day period from Edwards Air Force Base, California. In the lower stratosphere, the differences are related primarily to atmospheric changes over the two days. Above about 18 km, the differences are probably

related to measurement uncertainties and are about $\pm 5\%$ of the mean. A more conservative measure of the instrument performance is provided by summing the known uncertainties such as thermistor calibration, data transmission errors, optical detector drift and instrument outgassing^{11,23}. This method gives an uncertainty in mixing ratio of $\pm 15\%$. This is represented by the error bar in Fig. 1a. For the 14-yr record at Boulder, the standard deviation of the mixing ratio for levels above 18 km is about 0.3 p.p.m.v. about the mean of 4.1 p.p.m.v. or about 8% (Table 1).

For the purpose of trend analysis, the accuracy of the frost-point hygrometer is not as crucial as the precision of the measurements. At the same time as the soundings shown in Fig. 1a, a Lyman- α spectroscopic instrument was being flown on the ER-2 aircraft at the same location. The balloon-borne frost-point instrument gives stratospheric mixing ratios about 15% lower than the ER-2 Lyman- α instrument, which is typical of other such comparisons between frost-point and Lyman- α instruments. Preliminary laboratory comparisons suggest that the Lyman- α hygrometer may be biased slightly (about 10%) higher in its ER-2 flight configuration. Moreover, recent validation studies^{25,26} of the HALOE and MLS water-vapour sensors

TABLE 1 Observations of water vapour mixing ratios

Height (km)	Mean (p.p.m.v.)	Standard deviation (p.p.m.v.)	Number of observations	Trend (% yr ⁻¹)	95% confidence interval (% yr ⁻¹)
10–12	59.2	36.46	125	1.03	2.72
12–14	11.88	5.94	125	0.49	2.21
14–16	4.66	0.98	125	0.54	0.93
16–18	3.87	0.48	125	0.73*	0.55
18–20	3.85	0.35	124	0.84*	0.40
20–22	4.07	0.29	119	0.54*	0.32
22–24	4.21	0.29	114	0.38*	0.31
24–26	4.29	0.30	97	0.34	0.34

* Significant at 95% confidence level.

Mean, standard deviation, number of observations, linear trend and 95% confidence interval for water vapour mixing ratios over Boulder, Colorado, during the period 1981–94. The trends are computed for deseasonalized values. The 95% confidence interval is based on Student's *t*-distribution.

aboard the Upper Atmosphere Research Satellite show that there is excellent agreement ($\sim 5\%$ and $\sim 10\%$ r.m.s., respectively) between the stratospheric mixing ratios obtained with balloon-borne frost-point hygrometers and these two satellite instruments, verifying the quality of measurements with the frost-point instrument.

Figure 1b shows seasonal averages of mixing ratio profiles for water vapour over Boulder. In the lower stratosphere, there is a marked seasonal variation with lowest values in winter and spring, and highest values in summer and autumn, whereas in the region above about 18–20 km, the seasonal variation is small. In the upper troposphere, day-to-day as well as seasonal variability is large based on profiles obtained on consecutive days such as those from Edwards Air Force Base. The standard deviation is nearly equal to the mean in the upper troposphere at Boulder (Fig. 1b).

Although these measurements were made at a single site, we expect the stratospheric portion to be fairly representative of at least the mid-latitudes of the Northern Hemisphere. The average seasonal pattern in the stratosphere over Boulder is similar in both amplitude and timing to the latitude-mean water vapour mixing ratio for 30–40° N derived from SAGE II satellite data²⁷. The low variability in this altitude region also argues for control by large-scale processes, not local sources or sinks. In the lowest part of the stratosphere the seasonal cycle is prominent but it can largely be removed for trend determinations.

For each profile obtained, the data have been averaged over 2-km increments. The individual 2-km averages for eight stratospheric levels are shown in Fig. 2 with the mean for each level (dashed line). The solid line at each level represents a least-squares linear trend fitted to the deseasonalized 2-km-average mixing ratios for water vapour. The deseasonalized values are calculated by subtracting the 14-yr average for a month from each monthly observation, then adding the anomaly to the overall mean. The trend for levels from 10–26 km is summarized in Table 1 along with the mean, standard deviation and number of observations at each level. For heights between 16 and 26 km, there is an upward trend, significant at the 95% level. The largest trend ($\sim 0.8\%$ per year) is in the 18–20 km region. Lower down, there is also an increase with time, but the calculated trends are not statistically significant at the 95% level. At the highest level (24–26 km), there are about 25% fewer data points (usually because the balloon did not reach these altitudes) than at lower altitudes. The missing data are relatively evenly distributed over the measurement period. Below 16 km, the proximity to both the tropopause and hygropause makes the water vapour concentration much more variable, and the trend is not significant.

As methane builds up in the atmosphere¹⁵, it is expected that water vapour concentrations in the stratosphere should increase with time through methane oxidation¹⁴. Although rigorous modelling of the stratospheric chemical conversion of methane to water vapour²⁸ is beyond the scope of this investigation, we can

make an estimate of the expected increase. As pointed out by LeTexier *et al.*²⁸, the assumption of the direct conversion of one methane molecule to two molecules of water vapour is not strictly correct, because molecular hydrogen is also produced and this varies with latitude, season and altitude. The net yield when the oxidation of hydrogen is also considered, however, seems to be close to two. The use of a yield of two water molecules will provide a reasonable upper limit. From the long-term increase of methane of $\sim 0.7\% \text{ yr}^{-1}$ (refs 15, 29, 30; updated data after 1992 were also used to estimate the long-term global methane trend and are available from NOAA/CMDL), a concentration of methane in the lower stratosphere of 1.5 p.p.m., and the conversion of each methane molecule to two water vapour molecules, we estimate an upper limit of $0.02 \text{ p.p.m. yr}^{-1}$ or $0.5\% \text{ yr}^{-1}$. The observed change above about 20 km ($0.3\text{--}0.5\% \text{ yr}^{-1}$) is consistent with this estimate. The larger increases that are seen below 20 km may reflect additional mechanisms that are contributing to the trend especially in the lower stratosphere. The calculated trends are consistent with a warmer troposphere and hence a higher moisture content for air that could be exchanged between the troposphere and stratosphere^{31,32}. Because a byproduct of a warmer atmosphere could be an upward shifting of the profile (and usually higher mixing ratios) to higher altitudes, we also carried out the analysis at fixed pressure levels. The results were unchanged from those found using altitude layer averages.

The uncertainties in the mechanisms controlling the water vapour budget in the lower stratosphere, particularly in terms of exchange at the tropopause or other dehydration mechanisms^{10,33,34}, make changes related to tropospheric temperature increases difficult to determine. Aircraft flying in the upper troposphere and lower stratosphere may also have an effect, although this is likely to be more of a problem if higher-flying aircraft are developed³⁵.

Before the start of the programme at Boulder, a 15-yr set (1964–79) of frost-point hygrometer profiles was obtained at Washington DC^{11,23,36}. The instrument used was a predecessor to that used in Boulder but without modern solid-state electronics. There is more scatter in the earlier set of data (for example, the standard deviation at 20–22 km for Washington DC is 0.70 p.p.m.v. compared with 0.30 p.p.m.v. at Boulder). Although the mean value at stratospheric levels is nearly the same, the difference in variability makes it inadvisable to treat these two datasets as a single time series. No significant trends were found for the Washington DC series by itself, although trying to fit the data to a linear trend is misleading as an upward trend during the earlier part of the record is followed by a decline. Other earlier data from which a longer-term trend has been deduced come from the Meteorological Research Flights over England in 1954–55 and again in 1972–76 (ref. 13). These do not form a continuous record. The latter period shows a decline from beginning to end, but the increase from the earlier to the later series for the low stratosphere is about $2.4\% \text{ yr}^{-1}$, much larger than at the comparable altitude for the Boulder observations. Measurements in Australia from 1972 to 1979 using a balloon-borne radiometer¹² show a decline which seems to be consistent with other measurements made during this period^{11,13}. As it is expected that methane has been increasing throughout the period, the shorter-term variations are most probably associated with short-term climate variability. The data presented here seem, however, to be the best suited to detecting long-term changes.

The detection of an increase with time of water vapour in the stratosphere over Boulder adds to the concerns about the build-up of greenhouse gases and their effects on atmospheric chemistry as well as climate. Although the measurements reported here are at a single location, they are expected to be representative of the stratosphere over the highly populated northern mid-latitudes. Furthermore, these measurements indicate that a carefully executed monitoring programme using fairly simple, in-

expensive instrumentation can detect the expected changes in stratospheric water vapour. Such measurements at five global locations, for example the sites of the Network for Detection of Stratospheric Change³⁷, together with future satellite systems, are needed to address the issue of future global changes in stratospheric water vapour associated with methane increases and tropospheric warming. □

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Circulation in the glacial North Atlantic inferred from grain-size measurements

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RECORDS of nutrient proxies in marine sediments indicate that the nutrient distribution—and hence circulation—of the glacial North Atlantic Ocean was markedly different from that of today^{1,2}. But these tracers are influenced by several biogeochemical factors unrelated to ocean circulation^{3–6}, and thus do not provide a direct measure of the vigour of circulation. Distributions of grain size in marine sediments can provide such information, owing to the sorting effects of currents^{7,8}, if the characteristics of the input sediment flux are known. Here we present a record, inferred from grain-size measurements, of variations in the relative strength of deep and intermediate currents in the eastern North Atlantic over the past 25,000 years. We find that glacial intermediate water flowed rapidly at depths of between 1,100 and 2,000 m. In contrast, deep-water circulation was sluggish during the last glaciation, but increased in strength shortly after the glacial maximum. This would have resulted in increased heat flux to high latitudes, and may have triggered sudden deglaciation. Deep current strengths declined again at the start of termination stage IA and during the Younger Dryas, perhaps as a result of iceberg discharges (the so-called Heinrich events^{9,10}). A remarkably similar record of grain-size variations has been found in the western North Atlantic¹¹, indicating that these changes in circulation are ocean-wide.

The conveyor-belt system of North Atlantic Deep Water produced in the Norwegian and Greenland seas and compensating surface return of the North Atlantic drift exercises an important influence upon regional climate via the transport of heat from low to high latitudes¹². At present, sub-thermocline waters of the North Atlantic are nutrient-depleted due to the sinking of surface waters to form North Atlantic Deep Water (NADW). However, both carbon isotopes^{1,4,13} and Cd/Ca ratios² in benthic foraminifera indicate that the glacial water column was highly stratified, with nutrient-depleted intermediate waters at depths shallower than 2,000 m overlying nutrient-enriched waters. This

evidence has been interpreted as showing that the glacial North Atlantic was vigorously ventilated at intermediate depths by surface waters sinking in the north². These conclusions are not unequivocal, however, because of changes in the preformed nutrient content of surface water, atmosphere–water CO₂ equilibrium³ and carbon inventory⁴. There are also questions concerning the reliability of benthic foraminifera as recorders of bottom-water chemistry because of rapid seasonal influx of phytodetritus⁵ and dissolution⁶.

As an alternative to chemical tracers of water-mass evolution, we employ here a more direct physical proxy of ocean circulation, based on the grain-size characteristics of marine sediments. The mean size of the non-cohesive silt fraction (10–63 µm) is used as an indicator of relative current strength because bottom currents size-sort coarse silt during events of resuspension and ensuing deposition. Stronger currents yield a coarser mean size of the non-cohesive silt fraction, acting through both selective deposition and winnowing⁸.

The cores examined in this work are from the UK Biogeochemical Ocean Flux Study (BOFS) sites, between 50–60° N and 15–25° W, and range in depth from 1,100–4,045 m (Fig. 1). Reliable age control was achieved through accelerator mass spectrometry ¹⁴C dating of core BOFS 5K, and correlation to the rest of the suite via oxygen- and carbon-isotope profiles, magnetic susceptibility, planktonic foraminiferal species abundance, carbonate content, volcanic ash peaks and X-radiographic characteristics^{9,14} (Fig. 2). Grain-size analysis was carried out on the non-carbonate fraction (opal is <1%). Textural parameters have previously been related to bottom-water circulation both in the modern ocean⁷ and in palaeoceanographic investigations^{11,15,16}. But application of the technique has been limited, largely because of presumed difficulties with unknown source functions¹⁷. The glacial northeast Atlantic received variable input of ice-rafted detritus, which might cause changes in mean grain size independent of variation in bottom currents. We approach this problem by identifying the characteristics of the input flux as a function of time at a site which is unaffected by current winnowing or focusing. Grain-size changes in sortable silt from current-affected sites are calculated by difference from the input flux time-series (Fig. 2). The resulting variation in differential grain size is attributed to fluctuating current speed. To define the input flux, we use core BOFS 6K from the East Thulean rise which is away from the path of major currents and separated from the Rockall plateau by the South Rockall gap

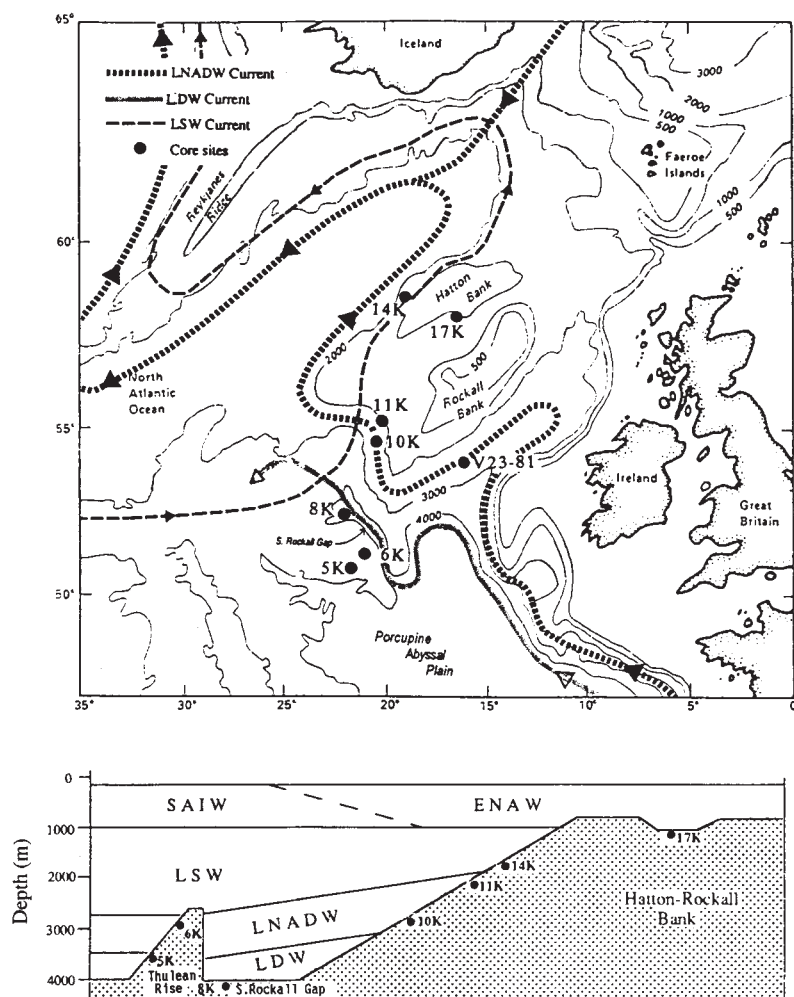


FIG. 1 Top, location of BOFS core sites and core V23-81²⁰. The paths of major deep-water currents are shown as dashed and dotted lines. Bottom, schematic cross-section (approximately south-north) illustrating present-day water-mass distribution in the Rockall area. These water masses are: the Lower Deep Water (LDW) of McCartney¹⁸, a derivative of Antarctic Bottom Water (AABW) which is warmer than AABW but which has higher Si and nutrient content than northern source water. Above this lies the familiar sequence of lower North Atlantic Deep Water (LNADW), Labrador Sea Water (LSW or upper North Atlantic Deep Water, UNADW), and shallower volumetrically insignificant intermediate waters (Sub-Arctic Intermediate Water and Eastern North Atlantic Water, SAIW and ENAW, respectively)¹⁹. Indicated flow paths are taken from McCave and Tucholke³⁰ and McCartney¹⁸.

(Fig. 1). The input flux at BOFS 6K shows a fairly constant low mean grain size during the glacial period and early deglaciation, rising by $\sim 3 \mu\text{m}$ during termination IB (Fig. 2a).

Cores from BOFS sites 8K and 10K are at depths of 4,045 m (under Lower Deep Water¹⁸, LDW) and 2,777 m (under lower North Atlantic Deep Water¹⁹, LNADW), respectively, on sediment drifts (Fig. 1) and have the potential to monitor changes in the strength of deep-water currents. Values of differential grain size for these cores are markedly similar, and reveal significant downcore variation, implying past fluctuations in current strength (Fig. 2b, c). Covariation of the silt mean records from present-day LDW- and LNADW-influenced sites suggests either a strong link between the speed of the two water masses or movement of the boundary between them so that both sites were bathed by the same water. Chemical data suggest that the top surface of the LDW was at $\sim 2,000$ m depth in this area during the last glacial^{20,21}, putting both sites under glacial LDW.

The flow of glacial LDW was sluggish at site 8K during the glacial period. The area is so far from the Antarctic Bottom Water (AABW) source that it is not possible to make inferences

about the southern source strength from our data. However, data from regions closer to the AABW source suggest slower flow during the glacial than at present^{15,22}. Our records from both core 8K and 10K indicate a marked increase in flow speed between the glacial maximum and the onset of deglaciation, peaking at 15–16 kyr ago. The record of grain-size variation on the Blake outer ridge in the western North Atlantic, is remarkably similar¹¹ (Fig. 2e), suggesting that the event is of ocean-wide significance.

Core V23-81 from $\sim 2,400$ m depth in the Rockall region (Fig. 1) shows a maximum in $\delta^{13}\text{C}$ coincident in time with the inferred pulse in deep current speed before termination IA (Fig. 2d)²⁰. This suggests an increase in the input of nutrient-depleted water of northern source. The inferred increase in the flux (product of thickness and speed) of northern source water through South Rockall gap could have been caused by increased production rate as speed at site 10K increased. The increase in LDW flow speed occurred because the two water masses are linked in geostrophic flow to the west through South Rockall gap^{23,24}. In the western basin, glacial intermediate water extended down to 2,500 m depth, but present-day NADW goes down to $\sim 4,300$ m (refs 2, 11). It is possible that the region below 3,000 m on Blake outer ridge was also still under glacial LDW at this time, but alternatively at least the shallower Blake outer ridge stations may have directly recorded the LNADW pulse. On Blake outer ridge, the present LDW and NADW both flow to the south²⁵. The deep gyres of the western basin are partly driven by Gulf Stream thickness variations²⁶, and an increase in Gulf Stream flux may have driven a more vigorous deep circulation of both glacial LDW and LNADW. An increase in the Gulf Stream flux would also have contributed the source water which cooled north of the late glacial polar front, probably in the Norwegian Sea, yielding the pre-termination pulse of deep water. The significance of this higher-speed pulse is that the surface waters which fed it would have contributed heat to northern regions and possibly have been the trigger for the deglaciation which immediately followed it, starting in the Nordic area²⁷. At this time insolation was increasing in the Northern Hemisphere but decreasing in the south, yet there was a brief period of marked retreat of mountain glaciers in the Andes and New Zealand just before termination IA¹², coinciding with the pulse in LNADW current speed shown here. Control

by deep circulation may well be involved.

After the start of termination IA, inferred flow speeds at sites 8K and 10K abruptly decreased and reached a minimum between 13 and 14 kyr. A pronounced minimum in $\delta^{13}\text{C}$ values at 13–14 kyr (during the deglaciation) in core V23-81 suggests reduced influence of northern source water. This abrupt decline is coincident with Heinrich ice-rafting event 1 (refs 9, 10), which introduced fresh water to the surface ocean through iceberg melting, decreasing the density of surface water by an amount probably sufficient to cause cessation of deep convection. Following this event, both differential grain size and $\delta^{13}\text{C}$ value increased, indicating a return to more vigorous deep-water circulation during the Allerod warming (~ 12 kyr ago). Current vigour apparently declined temporarily at the start of the Younger Dryas at the depth of core 10K, and this is matched by a minimum in $\delta^{13}\text{C}$ values in V23-81, again perhaps caused by a buoyant meltwater cap²⁸.

At shallower depths, differential grain sizes in cores BOFS 11K (2,004 m), 14K (1,756 m) and 17K (1,100 m) demonstrate faster current speeds in the glacial and Younger Dryas than in

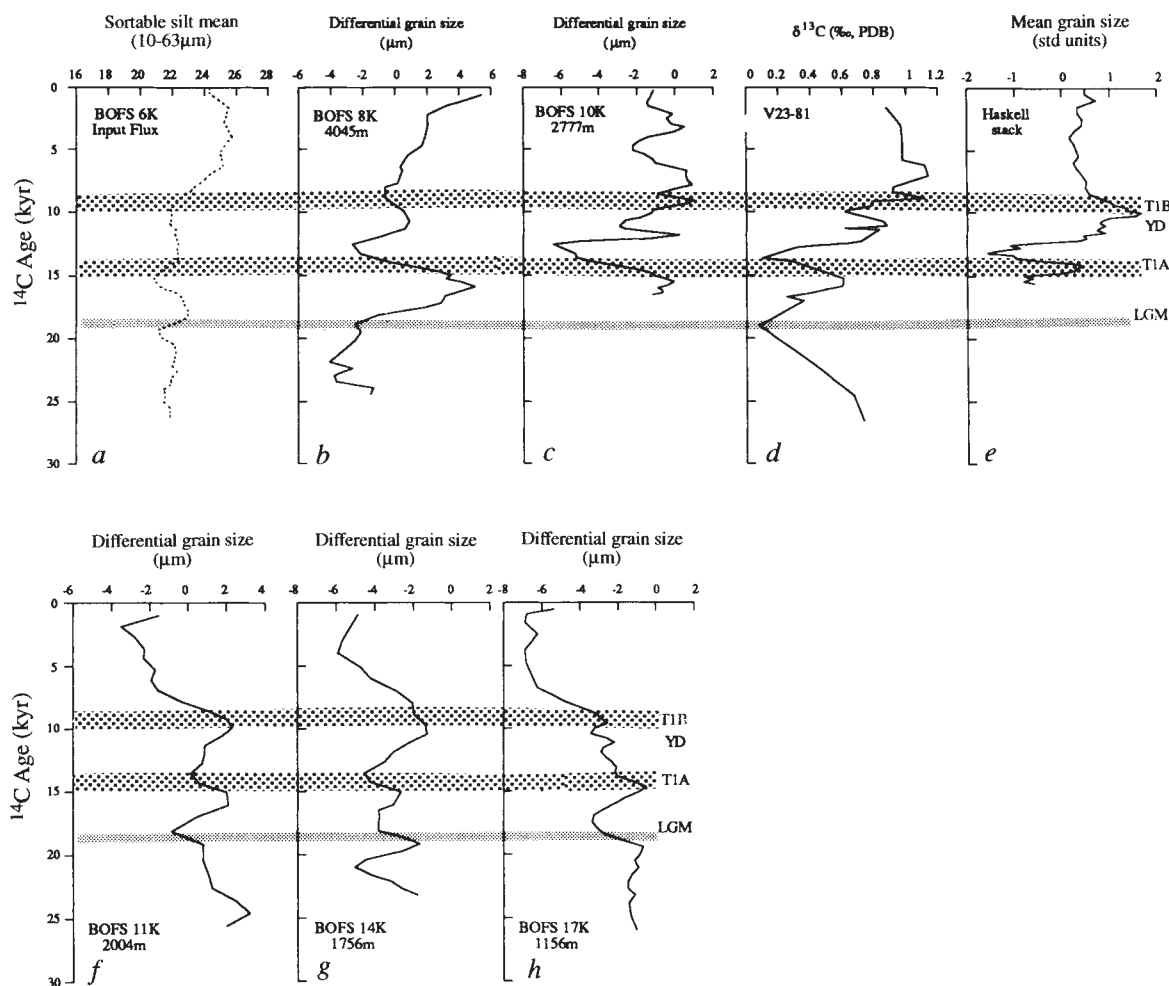


FIG. 2 Grain-size and carbon-isotope records in North Atlantic cores. Ages are ^{14}C (Libby) years, corrected for the reservoir effect by subtracting 400 years. The position of terminations IA and IB (TIA and TIB) are shaded, and the Younger Dryas (YD) and Last Glacial Maximum (LGM) are also indicated. a, 10–63 μm non-carbonate silt mean size as a function of time in core BOFS 6K, representing the input flux unmodified by current activity. The profile is smoothed with a three-point moving average. b, c, 10–63 μm silt mean grain size in BOFS cores 8K (b) and 10K (c), expressed as difference from the input flux in core 6K (differential grain size). d, Benthic $\delta^{13}\text{C}$ record (*Cibicides wuellerstorfi*) in Core V23-81²⁰. e, Weighted-average mean grain size (in units of standard

deviation about the mean) of 6–63 μm non-carbonate silt in cores CH88-7P, 10P and 11P⁹ from the Blake outer ridge (30° N, 74° W), for which age control is achieved by correlation with core KN31-GPC-5³¹. f–h, Differential grain size (as defined for b and c) of the 10–63 μm silt in intermediate-depth cores BOFS 11K, 14K and 17K. (Some of the data on which the correlations are based is in ref. 9 (magnetic susceptibility and $\delta^{18}\text{O}$ *Bulloides* for some cores) but all data may be obtained from the authors on request. Note that many of these data are on the BOFS database on CD-ROM from the British Oceanographic Data Centre, Proudman Lab., Bidston, Merseyside.)

the Holocene. This is consistent with carbon-isotope and Cd/Ca data, which indicate nutrient-depleted glacial waters at these depths^{2,21}. Covariation of grain-size records in these cores implies that all three sites were subject to the same flow regime during the glacial period. There is also a pronounced decline in speed at all three sites during the Holocene (from a high just before termination IB at 11K, 14K and Blake outer ridge) (Fig. 2e–h).

These grain-size records provide direct evidence for changes in the vigour of palaeocirculation which is independent of isotopic and chemical indicators. Temporal patterns of circulation reassuringly similar to some of those inferred from isotopic and chemical tracers are demonstrated. At a time when the reliability of benthic geochemical data is in question^{5,29}, our method provides direct information on the physical oceanographic changes driving geochemical variation. □

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Continental crust composition constrained by measurements of crustal Poisson's ratio

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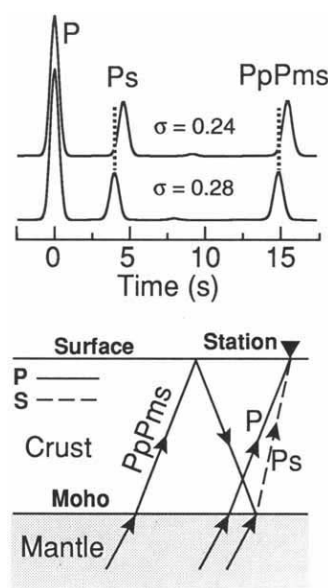
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DECIPHERING the geological evolution of the Earth's continental crust requires knowledge of its bulk composition and global variability. The main uncertainties are associated with the composition of the lower crust. Seismic measurements probe the elastic properties of the crust at depth, from which composition can be inferred. Of particular note is Poisson's ratio, σ ; this elastic parameter can be determined uniquely from the ratio of P- to S-wave seismic velocity, and provides a better diagnostic of crustal composition than either P- or S-wave velocity alone¹. Previous attempts to measure σ have been limited by difficulties in obtaining coincident P- and S-wave data sampling the entire crust². Here we report 76 new estimates of crustal σ spanning all of the continents except Antarctica. We find that, on average, σ increases with the age of the crust. Our results strongly support the presence of a mafic lower crust beneath cratons, and suggest either a uniformitarian craton formation process involving delamination of the lower crust during continental collisions, followed by magmatic underplating, or a model in which crust formation processes have changed since the Precambrian era.

Estimates of the bulk composition of the Earth's crust are limited by the uncertainty in the composition of the lower crust. A variety of approaches, including seismic, crustal xenolith and isotopic studies, as well as direct examination of exposed crustal sections, have led to various estimates of lower-crustal compositions ranging from felsic to mafic^{2–5}. Seismic measurements, perhaps the most direct method for probing the lower crust, have been impeded by the non-uniqueness of compressional-wave velocities (v_p) for the lower crust where both higher metamorphic grade and increasing mafic content can have similar effects² on v_p . The non-uniqueness is partially alleviated by the addition of shear-wave (v_s) data to estimate v_p/v_s (ref. 1), and hence Poisson's ratio, σ . For common rock types, σ varies from 0.20 to 0.35 and is particularly sensitive to composition; increasing the silica content lowers σ and high mafic content increases it (ref. 6). For lower-crustal rocks, low σ (<0.26), intermediate σ (0.26–0.28) and high σ (>0.28) are characteristic of felsic, intermediate and mafic compositions, respectively². Although the importance of σ has been recognized for at least two decades¹, a comprehensive, 1992 review paper² cited only 11 reliable *in situ* measurements of Poisson's ratio. We use a simple teleseismic method, and data from the growing global network of digital broadband seismograph stations, to increase the number of estimates of Poisson's ratio by a factor of seven.

We measure the travel times of converted waves in the P waveforms of a teleseism recorded at a single seismic station to estimate the v_p/v_s of the crust beneath the site. A teleseismic P-

FIG. 1 Bottom, geometry of ray paths for the P wave, the converted wave Ps, and multiple converted wave PpPms generated by a teleseismic plane wave interacting with the impedance contrast at the base of the crust. Top, synthetic receiver functions for a crust with Poisson's ratio of 0.24 (upper trace) and 0.28 (lower trace). We calculate v_p/v_s (then σ) from the ratio of differential times (Ps–P)/(PpPms–Ps) and estimate the crustal thickness using the Ps–P time and the estimated value of v_p/v_s for a range of average crustal P-wave velocities. The main assumption in this method is that the Ps and PpPms phases propagate in a laterally uniform crust over the distance sampled by the phases (20–50 km). A tangential receiver function is non-zero only in the presence of lateral heterogeneity and is used to assess the quality of measurements made using the radial receiver function. We minimize the effect of lateral heterogeneity on our result and enhance the multiples by using data with periods longer than 3–5 s.



wave incident on the base of the crust generates a converted S-wave (Ps) and a converted P-wave multiple (PpPms) within the crust⁷ (Fig. 1). The converted phases are isolated from source effects by deconvolving the vertical from the radial component of the P waveform to produce a time series called a receiver function^{8–10}. The ratio of the Ps–P time and the PpPms–Ps time is directly related to v_p/v_s , independent of crustal thickness but with a slight dependence¹¹ on v_p ; for a range in v_p of 6.0–6.75 km s^{–1}, the change in v_p/v_s is at most 0.05 and the change in σ is no more than 0.02. Our approach is similar in principle to another single-station teleseismic technique¹². This other method uses the redundancy of multiple phases within one seismogram, but our method does not require computation of synthetic seismograms so making it suitable for a rapid global survey. As a test of our methodology, we compared our measurements with those from a coincident seismic refraction study. Both compressional- and shear-wave Moho reflections were recorded in a recent seismic refraction experiment in eastern Ontario and northern New York state¹³. The ratio of the S-wave and P-wave travel times indicates a Poisson's ratio of 0.28 ± 0.01 for the crust in the southeastern Precambrian Grenville province¹⁴. We

TABLE 1 Poisson's ratio and crustal thickness by crustal type

Crustal type*	Vol. %†	No. obs‡	v_p § (km s ^{–1})	σ_{ave}	σ_{sd}	σ_m	h_{ave} (km)	h_{sd} (km)	h_m (km)
Shield	16	14 (0)	6.5	0.29	0.02	0.29	36.9	2.9	35.7
Platform	51	7 (3)	6.5	0.27	0.03	0.26	41.5	6.8	40.7
Pal.	19	17 (4)	6.5	0.27	0.03	0.27	33.4	5.6	33.4
M-C	14	24 (2)	6.25	0.25	0.04	0.24	32.6	6.8	31.1
IA	—	4 (1)	6.75	0.31	0.05	0.33	28.5	3.6	27.6

σ , Poisson's ratio; h , crustal thickness.

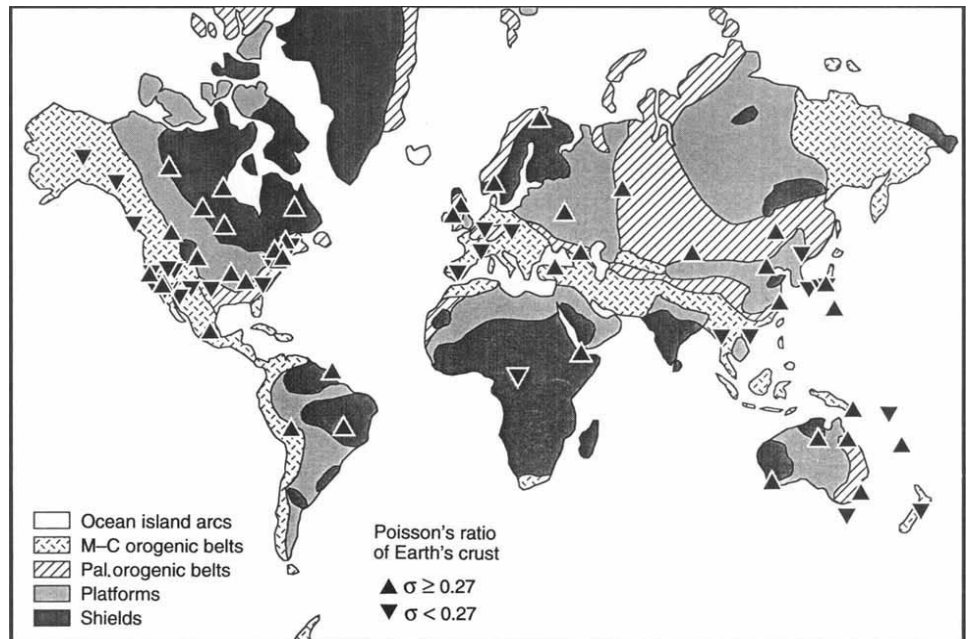
* Defined in Fig. 2 legend.

† Normalized continental crustal volume percentage calculated from surface area percentage¹⁶ and our estimated average crustal thicknesses.

‡ Number of measurements used to calculate average (subscript ave), standard deviation (sd), and median (m). Numbers in parentheses are measurements truncated from calculation because they were more than ± 0.07 from the initial average.

§ Average crustal velocity used in the calculation of Poisson's ratio and crustal thickness. Estimates taken from global compilations of seismic refraction and reflection studies²⁹.

FIG. 2 Global variation of Poisson's ratio measured at digital broadband seismograph stations accessible through international, national and regional data centres. The crust is divided into five categories^{16,22}. Shields are areas of exposed Precambrian-age crust, usually found in the interiors of the major continents. Platforms (Plat.) are shield-like crust with sedimentary cover 1–5 km thick. Orogenic belts are curvilinear regions of crust that have experienced one or more Phanerozoic-age tectonic events. Palaeozoic (Pal.) orogenic belts are relatively stable with eroded mountains lacking significant crustal roots. Mesozoic–Cenozoic (M–C) belts are often still tectonically active, with considerable surface relief and crustal thickness variations. Island arcs (IA) form at ocean–ocean convergence zones and are regions of oceanic crust thickened by magmatic additions. Continental volcanic arcs are included in the M–C orogenic belts.



determined a similar Poisson's ratio of 0.27–0.28 from receiver functions at RSNY, the closest broadband station to the seismic refraction study. Our measurement is also consistent with an independent teleseismic estimate for eastern Canada of $\sigma > 0.33$ for the lower crust with an average crustal value¹⁵ of 0.27.

We examined receiver functions at 114 stations with data obtained electronically from a world-wide group of data collection organizations, and reliable estimates were made at 76 stations. At the remaining stations, either a crustal multiple could not be identified or the receiver functions were judged to be too complicated for a survey study. The distribution of global seismic stations (and hence the sampling of the crust) is heavily weighted towards North America and continental Mesozoic–Cenozoic orogenic belts, although we have some data from all the major continents except Antarctica. We separate the measurements on the basis of crustal type, namely shields, platforms, Palaeozoic orogenic belts, Mesozoic–Cenozoic orogenic belts, and ocean island arcs¹⁶ (Fig. 2). Shields and platforms are tectonically stable and are often grouped together as cratons¹⁶. Based on this five-category crustal classification, an interesting pattern emerges from the σ measurements (Table 1; Fig. 3). Precambrian shield crust has consistently high values of σ , averaging 0.29 ± 0.02 . platform values are more variable but average 0.27 ± 0.03 . The average shield crustal thickness is 37 km and platform areas average ~ 42 km. The lower σ and 4–5 km additional thickness of the platform crust probably represents the effects of sedimentary cover rich in silica. Quartz has an extremely low σ (0.09) and a thick clastic sedimentary section will lower the average σ and increase the average crustal thickness. Palaeozoic orogenic belts are characterized by Poisson's ratios with a 0.27 average and median, and a crustal thickness average of 33 km. Measurements from the Mesozoic–Cenozoic regions of active or recent tectonics are variable, but in general lower, averaging about 0.25 ± 0.04 for σ , and 33 ± 6.8 km in thickness. We believe that the large standard deviation in both σ and thickness for the Mesozoic–Cenozoic crust reflects, in part, the variety of tectonic terranes encompassed by this broad classification. However, a more detailed classification is not yet justified by the number of available measurements. Additional, but less well-constrained, measurements along some island arcs of the southwestern Pacific have very high σ , averaging 0.31 ± 0.05 and crustal thickness average of 28.5 km. By weighting the averages of each crustal type by the volume percentage

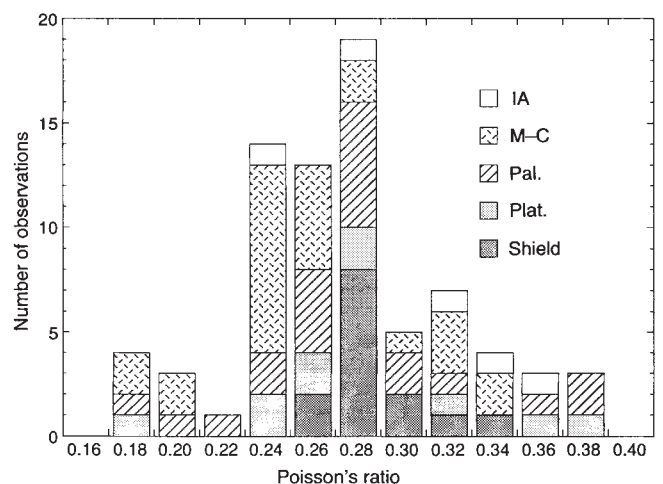


FIG. 3 Stacked histogram for all measurements of Poisson's ratio, sorted into 0.02 bins and stacked by crustal type (see Fig. 2 for definitions of crustal types).

of each type (Table 1), we estimate that the average Poisson's ratio for the bulk continental crust is 0.27, consistent with an intermediate composition. There is a significant number (18) of outlier measurements with values < 0.23 or > 0.33 (Fig. 3). We suspect that some of these are biased by complex structure, although there is independent evidence for unusually low Poisson's ratio in some thickened crust¹⁷.

Our most robust observation is the high σ (0.29) for shield crust. The upper crust of shields has been estimated from numerous studies to have a felsic-to-intermediate composition^{18,19}. Based on this composition and available measurements, we estimate the Poisson's ratio of the upper crust is ≤ 0.26 . If the bulk value of the entire crust is > 0.28 , the lower crust must have a value of ~ 0.30 . This range of Poisson's ratio and high v_p for the lower crust² is indicative of a mafic composition, the presence of water or another volatile component under high pore pressure, or both^{15,20}. High pore pressure also significantly decreases the velocity of both P and S waves²¹. The observed

high v_p of cratonic lower crust and our observation of high Poisson's ratio strongly suggests a mafic composition rather than high pore pressure. With our limited data, we observe no systematic difference between Archaean and Proterozoic crust, in contrast to some previous studies²².

We interpret the low Poisson's ratio (0.25) for Cenozoic–Mesozoic crust as indicating a predominantly felsic composition. The higher values for older crust are interpreted as reflecting a more intermediate-to-mafic composition. Specifically, Precambrian cratons have a bimodal composition distribution with a felsic-to-intermediate upper crust and a mafic lower crust. These conclusions have several possible implications for continental crustal evolution. Two main processes have been invoked in continental growth: the amalgamation of island arcs onto the edges of pre-existing continents, and pervasive intrusion and underplating of magmas derived from the upper mantle²³. Both processes add predominantly mafic components to the crust^{24,25}. The felsic composition of young orogens then implies that if magmatic addition occurs during orogenesis, either a delamination-type process must operate to remove the mafic component²⁴, or there is little new crust added by magmatic processes, and young orogenies mainly involve reworking of existing continental crust²⁶. If the orogeny involves remobilization of an older and more mafic crust, again, some refining process such as delamination must operate to produce a predominantly felsic crust. If delamination is a common process, it raises the question of why older crust is more mafic.

There are two endmember scenarios. In a uniformitarian model, continental crust grows by amalgamation of island arc (mafic) terranes but evolves toward a more intermediate composition, first by delamination of a mafic lower crust during continental collisions. Then the remaining felsic crust is stabilized with a more mafic composition by pervasive underplating of mantle-derived magmas²⁷. In this scenario young orogens, which have a felsic composition, involve crust that has not yet evolved to completion. Alternatively, the Precambrian cratons may have evolved in an entirely different manner, by a process that generated an initially more intermediate-to-mafic crust that stabilized by developing a refractory mantle root close to the time of crust formation²⁸. In this scenario, processes in young orogens are fundamentally different from the processes active in the Precambrian; young orogens involve recycling and refining of existing continental crust with little crustal growth²⁶. □

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Complex morphology of subducted lithosphere in the mantle beneath the Tonga trench

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At the Tonga trench, old Pacific sea floor subducts at a rapid rate below the Indo-Australia plate, generating most of the world's deep earthquakes (focal depth >300 km)^{1,2} and producing a deep slab of former oceanic lithosphere. The seismogenic part of the slab has been mapped in detail^{3,4}, but its fate has remained enigmatic. Here I present evidence from seismic tomography that the Pacific plate descends deep into the Earth's mantle along a trajectory that is more complex than previously thought. In the north, the slab deflects in the transition zone (between about 400 and 700 km depth) before continuing into the lower mantle (below 700 km). Further south, penetration into the lower mantle occurs without a kink. The slab morphology can be explained in terms of the recent tectonic evolution of the subduction system, and reconciles pre-existing evidence from this region for both local horizontal flow in the transition zone^{2–8} and slab penetration into the lower mantle^{9–12}.

In the southwest Pacific (Fig. 1a) the large age (>120 Myr) of oceanic lithosphere and the high rate of subduction (>10 cm yr⁻¹)¹³ combine to produce a deep, negatively buoyant slab of former oceanic lithosphere. This slab was the subject of pioneering work on deep seismicity that further substantiated the concept of plate tectonics^{2,5,6}. Analyses of travel times of seismic waves revealed fast P-wave propagation below the deepest earthquakes, suggesting the continuation of the slab into the Earth's lower mantle^{9–12}. On the other hand, locations and focal mechanisms of deep earthquakes indicate internal deformation and horizontal flow in the transition zone^{2,9,14} which has been interpreted as evidence against the continuation of the slab below 700 km depth, or for the detachment of the slab^{7,14,15}.

In a recent tomographic study R.v.d.H. and E. R. Engdahl (manuscript in preparation) used nearly 10⁶ travel times of first and later-arriving compressional waves to map mantle structure to a depth of 1,600 km below the Fiji–Tonga region. Earthquake locations and phase data resulted from nonlinear hypocentre relocation and phase re-identification using arrival times from the International Seismological Centre^{16,17}. Results of this investigation confirm some inferences from an earlier study¹¹, but the new images are of improved quality owing to better (and more) phase data and hypocentres, denser sampling due to inclusion of later-arriving phases, and the use of the iasp91 reference model^{16,18}. Moreover, they are given a different interpretation. Here I focus on the relationship between large-scale slab structure and lateral displacement of the Tonga subduction system. Mantle structure further north is complex, owing to the subduction of the New Hebrides basin beneath the clockwise-migrating

FIG. 1 *a*, The southwest Pacific study region (Cartesian map projection). Shading indicates water depth (dark, deep; light, shallow). Dashed line, boundary between the Pacific and Indo-Australia plates; white dots, epicentres of earthquakes (after E. R. Engdahl, R.v.d.H. and R. Buland, manuscript in preparation) with Richter scale magnitude >5.2 (small dots, focal depth <70 km; large dots, depth >300 km). Triangles, sites of active (Holocene–present) arc volcanism. Lines labelled '3a', '3b' and '3c' give the position of the mantle cross-sections displayed in Fig. 3. *b*, Approximate position of the Tonga–Kermadec trench at intermediate stages (40 Myr ago, 30 Myr ago, and present day) during the post-Eocene clockwise migration of the Tonga trench (after Walcott³⁸). This rotation is superimposed on possible northward motion of the shallow part of the subduction system relative to the deeper mantle^{8,9}. Abbreviations used: SFB, South Fiji basin; NFB, North Fiji basin; LB, Lau basin; TKR, Three Kings rise; PP, Pacific plate.

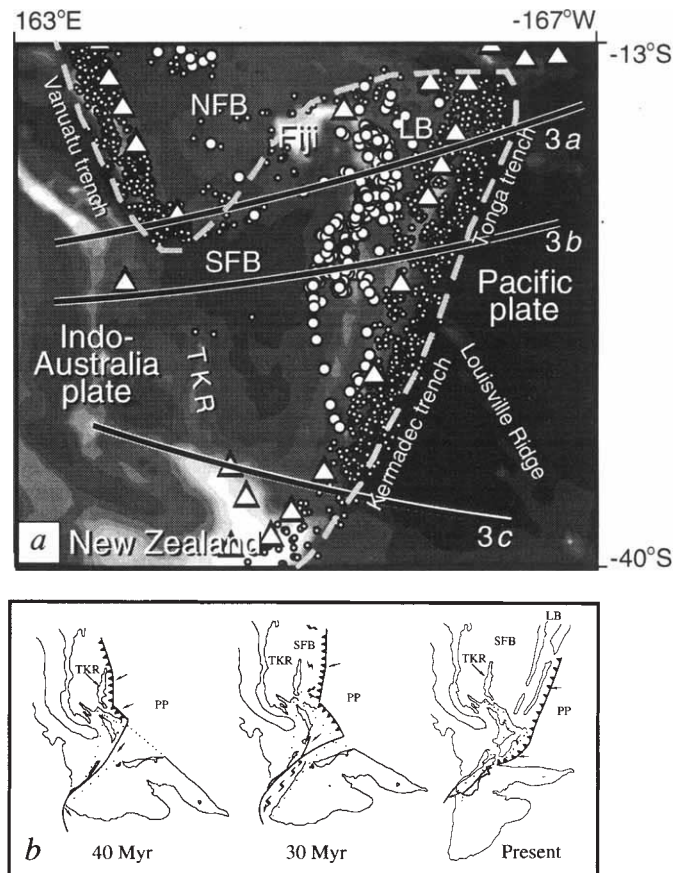


FIG. 2 Results of tomographic inversions R.v.d.H. and E. R. Engdahl, manuscript in preparation). *a*, Lateral variation in P-wave velocity below the study region at ~ 300 km depth. Regions of fast wave propagation are depicted by shading in order to emphasize slab structure. Seismicity is depicted by white dots. At this depth, P waves in the slab propagate up to 4% faster than average. *b*, Spatial distribution of ray paths used in the inversion. *c*, Recovery of a synthetic model consisting of velocity anomalies with a horizontal dimension of $0.9^\circ \times 0.9^\circ$ ($\sim 95 \times 95 \text{ km}^2$ at this depth). Using the ray distribution as in the actual data inversion, synthetic data were generated from this model and inverted with the objective of recovering the input model⁴². The position of the synthetic anomalies is recovered near the slab but the amplitude of the input signal is not well resolved. Resolution degrades with increasing distance away from the slab owing to irregular sampling. Artificial errors were not added to the synthetic data. The regular pattern of the input model is depicted in the inset; the recovery of this pattern is displayed in the main part of the figure. *d*, Velocity anomaly (up to 2% fast) in the lower mantle at a depth of ~ 900 km. *e*, Sampling at 900 km depth. *f*, Synthetic anomalies used to test resolution of lower-mantle structure have a horizontal dimension of $1.8^\circ \times 1.8^\circ$ ($\sim 170 \times 170 \text{ km}^2$ at 900 km depth) (inset), and are adequately recovered in a large region around the structural feature depicted in *d*. Spatial resolution degrades to the east of the slab. The variation in wave speed is relative to the iasp91 model; the sampling is given as the number of ray paths, propagating through a particular part of the model.

METHODS. The images *a*, *c*, *d* and *f* resulted from a damped linearized inversion^{16,42} of travel-time data of first-arriving P waves, that is compressional seismic waves propagating directly from source to

receiver, and the later-arriving depth phases (pP and pwP), which travel initially upwards from the source and reflect at the surface of the Earth (water-sediment/water-atmosphere interface for pP/pwP). The data were obtained from a nonlinear relocation and phase re-identification procedure using arrival times published by the International Seismological Centre (ISC) and ISC hypocentres as initial values (E. R. Engdahl, R.v.d.H. and R. Buland, manuscript in preparation). The combination of P, pP, and pwP data improves the sampling of structure and the constraints on earthquake focal depth, and reduces the trade-off between hypocentre mislocation and aspherical variations in seismic velocity^{16,17}.

Vanuatu trench. Further south, westward subduction beneath the North Island of New Zealand began only recently (<10 Myr ago), is probably unrelated to the deep slab discussed here, and the evolution of the southern Kermadec arc is still controversial (R. Walcott, personal communication).

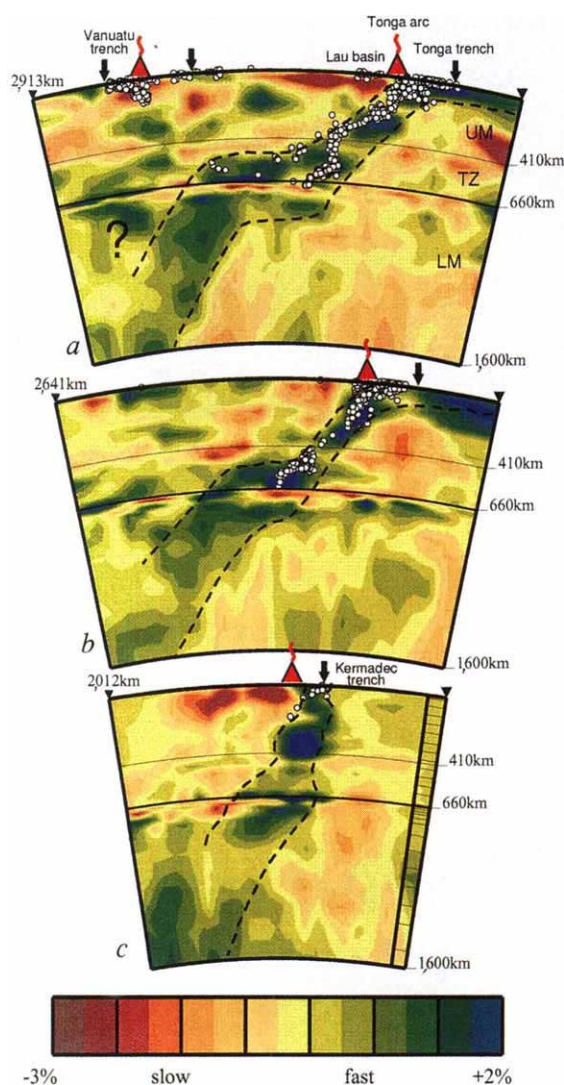


FIG. 3 Tomographic images of aspherical variations in P-wave velocity in the mantle below northern Tonga (a), central Tonga (b) and Kermadec (c). See Fig. 1 for location of the cross-sections. Green-blue colours, mantle regions where P-wave velocity is faster than the iasp91 reference model¹⁸ at the same depth; white dots, earthquake hypocenters projected from a distance of up to 50 km on each side of the plane of cross-section. Black arrows (red triangles) mark the intersection of the line of cross-section with the plate boundary (volcanic arc). A prominent feature, schematically outlined by dashed lines, is the zone of fast seismic velocity dipping into the mantle at the trench and delineated by seismicity in the upper mantle (UM) and transition zone (TZ), and aseismic in the lower mantle (LM). Slow P-wave propagation is evident below volcanic arcs and regions of recent or active back-arc spreading such as the Lau basin, and also adjacent to the slab in the deeper mantle. This was also observed by Zhou¹¹. At large depths some of this signal is an artefact from the mapping of the fast slab (Fig. 4).

METHODS. In vertical direction, the dimension of the blocks used to parametrize the mantle volume under study varies from 35 km near the Earth's surface to 200 km in the lower mantle. One column of the block model is superimposed on the image at the right-hand side of Fig. 3c. Between 590 and 730 km depth, thin layers (~15 km) are used to detect possible rapid variations in P-wave velocity near the "660 km" discontinuity and to confine to a narrow depth range artificial structure due to inadequate representation of the discontinuity in the reference model.

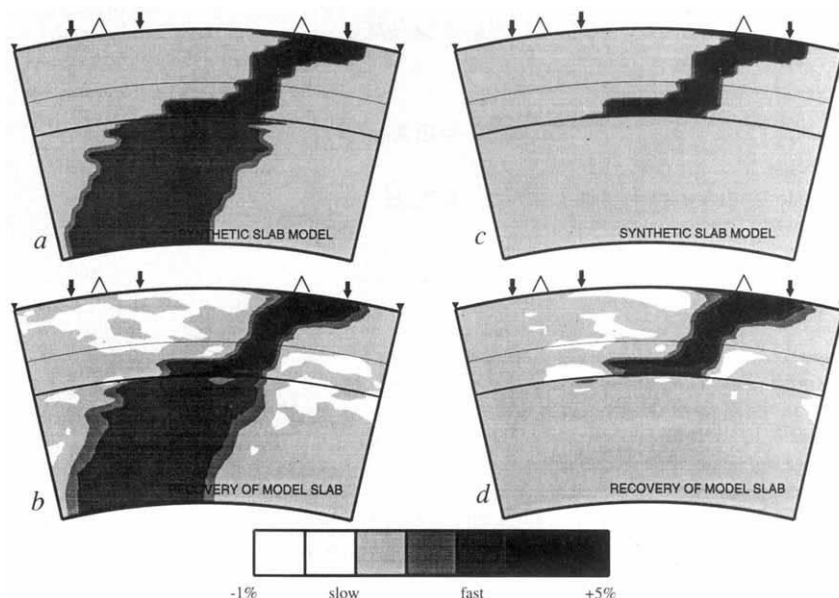
Seismic tomography images 'cold' slabs of subducted lithosphere as regions of fast wave propagation. Figure 2a shows a seismic velocity anomaly associated with the subducted Pacific plate in the upper mantle that is virtually parallel to the trench and delineated by seismicity. Although the sampling of mantle structure is irregular, it is dense near the feature interpreted as subducted lithosphere (Fig. 2b). Consistent with results of global imaging¹⁹, Fig. 2d reveals a seismic velocity anomaly in the lower mantle with an orientation that differs significantly from the strike of the present-day trench. Its position is not controlled in a trivial way by the distribution of the ray paths (Fig. 2e). The angle, measured in horizontal plane, between the orientation of the slab-like structures at depth of 300 and 900 km is ~35°.

As expected from the images in map-view, vertical sections across the trench reveal considerable variation in slab structure along the strike of the arc (Fig. 3). In the north, the Pacific plate descends into the upper mantle at an angle of ~50° and becomes almost horizontal in the transition zone and uppermost lower mantle (Fig. 3a). This deflection is consistent with inferences from seismicity^{2-9,14}. However, the slab is not trapped in the transition zone but sinks deeper into the lower mantle several hundred kilometres further west. The deep earthquakes below the Fiji basin, previously interpreted as evidence for detached parts of subducted lithosphere^{6,14,15}, are located in the continuous slab. Vertically below the inclined seismic zone, the slab is imaged to a depth of ~800 km. This is in agreement with evidence from residual sphere analyses of travel times^{9,10}, which may have detected only part of the complex slab structure. Towards the south, both the seismic zone^{3,4} and the slab become steeper, and the kink of the slab across the transition zone disappears (Fig. 3b, c).

In the upper mantle and transition zone, the lateral variation of slab morphology is well resolved (Fig. 2c) and is consistent with the geometry of seismic zones^{3,4} (Fig. 2a). To assess image reliability for depths beyond seismicity additional testing is required. Inversion of subsets of the data produces similar solutions demonstrating that the imaging is robust. In addition, the recovery of a synthetic velocity model indicates that the structural feature in the lower mantle (Fig. 2d) is well resolved (Fig. 2f). However, a slight degradation of the spatial resolution to the east leaves the possibility that the deep slab is actually broader than imaged in Fig. 3a. Its steep, eastern part can be overlooked if it is not sampled effectively by the ray paths used. The potential recovery of a broad slab is tested explicitly by inverting travel-time data computed from a model slab inspired by work of Fischer and colleagues^{9,10} (Fig. 4a). The adequate recovery of the model (Fig. 4b) demonstrates that the lower-mantle slab cannot be significantly thicker than depicted in Fig. 3a because this would have been detected in the actual inversion. Similarly, inversion of data computed from a synthetic upper-mantle slab model (Fig. 4c) demonstrates that the lower-mantle anomaly does not result from the smearing of structure at shallower depths (Fig. 4d). The low velocities just below 410 and 660 km depth (Fig. 3) are reference-model artefacts¹⁶ indicating that, locally, the increase in P-wave velocity across the discontinuities is either smaller or occurs at a larger depth than in the global iasp91 model. Low velocities near the slab between 660 and 710 km depth (Fig. 3) concur with the inference from P-to-S wave conversion that the endothermic, isochemical phase change from spinel to post-spinel structure that coincides with the "660 km" discontinuity may be depressed by up to 50 km owing to the presence of the cold slab²⁰.

To understand slab morphology in terms of mantle dynamics, one should realize that the shape of the slab is a snapshot of a time-dependent process influenced by relative plate motion²¹⁻²³. Lateral displacement of a convergent plate boundary relative to the deeper mantle can produce the observed kink in the subducting slab (Fig. 3) if slab descent is slowed down on approaching the lower mantle because of an increase in viscosity^{21,24,25} and/or effects of isochemical phase changes on buoyancy²⁵⁻²⁷. If trench

FIG. 4 Results of tests to investigate whether the deep slab can actually be broader than imaged (a, b), and whether the lower-mantle anomaly in Fig. 3a can result from the smearing of shallower structure (c, d). a, Model slab synthesized from the three-dimensional slab structure shown in Figs 2 and 3. In the upper-mantle and transition zone the slab is similar to that of Fig. 3a, but is artificially enlarged below 800 km depth. In the model slab, P-wave velocity is 4% faster-than-average for depths <300 km, 3% for 300–660 km, and 2% below 660 km; velocity perturbations are set to zero outside the slab. Synthetic travel-time data are computed from this model using the same ray distribution as in the actual data inversion. b, Recovery of the model slab. Both the shape of the model slab and the amplitude of the velocity anomalies are adequately recovered on inversion of the synthetic data. The assignment of only positive velocity anomalies to the model slab (a) produces a biased distribution of synthetic data. This bias is (partly) removed on least-squares inversion of the data, which produces slower-than-average wave propagation in some regions of the model space. c, Model slab with perturbations set to zero for depths greater than 660 km. d, Recovery of the model slab, showing that the shape is well recovered, although with some loss of amplitude.



Note that slab structure in the upper mantle and transition zone is not mapped into lower-mantle anomalies.

migration is fast compared to the sinking velocity, the slab is (temporarily) laid down atop the more viscous lower layer before it flows to greater depth²⁸. In contrast, the slab buckles without deflection if trench migration is slow compared to slab descent^{21,24,29}. I now argue that the former situation may apply to northern Tonga (Fig. 3a) and the latter to Kermadec (Fig. 3c).

Subduction processes controlled the Late Palaeozoic tectonic development of east Australia^{30,31}, but the Pacific/Indo-Australia plate boundary has been migrating away from the continent since the Cretaceous breakup of eastern Gondwanaland. This migration has been accompanied by the progressive eastward fragmentation of arc systems and by episodes of sea-floor spreading in marginal basins (Tasman sea, ~65 Myr ago; Fiji basin, 34–25 Myr; Lau basin, 5 Myr–present)^{30,37}. A change in Pacific-plate motion 40–45 Myr ago probably triggered the current episode of subduction below the Indo-Australia plate³⁷ along the Three Kings rise, an extinct arc^{35,36,38}. Subsequent changes in relative plate motion³⁷ caused a clockwise rotation

of the Tonga–Kermadec trench, back-arc spreading in the South Fiji^{35,38} and Lau basins^{32,35,39–41} (Fig. 1b), and the initiation of westward subduction beneath New Zealand³⁸. This rotation is superimposed on a possible northward motion of the plate boundary^{8,9} and is estimated from tectonic reconstructions at ~20° in the past 5 Myr and possibly twice as much since the Eocene epoch (Fig. 1b)^{38–41}. The rate of trench migration decreases from north (Fiji–Tonga) to south (Kermadec). The rotation angle is in excellent agreement with the change of the orientation of the slab with depth inferred from the tomograms (Fig. 2a, d).

Plate reconstructions and fluid-dynamical experiments provide powerful tools for the interpretation of slab morphology as revealed by seismic inversions, but causal relationships between slab deflection, trench migration and back-arc spreading are not yet completely understood. Quantitative modelling of this geodynamical system^{25,27}, along with continued seismological investigation, will improve our understanding of convective flow in the Earth's interior and its relationship to surface tectonics. □

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Heritability of a sexually selected character expressed in both sexes

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SEXUAL selection is thought to be responsible for the evolution of exaggerated male characters and of female mate preferences¹⁻⁷. Evolutionary mechanisms driven by an advantage to the progeny are only effective if the preferred character has a large genetic component of variance; in most systems in which sexual selection operates, little is known of the relevant genetics⁸. We have measured parent-offspring correlations, and report here that the preferred character (adult size) in seaweed flies has large additive genetic variance in males, but not in females. Virtually all the variance in male size is attributable to a chromosomal inversion system and, consequently, because this system is also a major determinant of larval viability^{9,10}, male size could be used by females as a reliable indicator of offspring survival.

It is now well established that the heritability of sexually selected characters expressed only in males is generally greater than the heritability of other traits subject only to viability selection (see ref. 11). For those characters expressed in both sexes, but subject to sexual selection only in males, theory predicts the evolution of differential expression of the relevant loci in the two sexes^{12,13}, and over considerable evolutionary time, the genetic correlation between the sexes could become negligible. There have been few tests of these predictions, but the heritabilities of tail length in barn swallows¹⁴ and of body size in dung flies¹⁵ do not differ significantly in males and females (although the estimates in barn swallows were based on only five pairs of females).

In seaweed flies (*Coelopa frigida*) females prefer to mate with large males¹⁶. Adult size in both sexes is influenced by a large chromosomal rearrangement (the $\alpha\beta$ inversion system on chromosome 1) which is also associated with larval survival^{9,10}. Because heterokaryotypic larvae exhibit superior viability, mate preferences based on size could generate fitter offspring, a slightly unusual type of 'good genes' female choice.

Using crosses done under controlled conditions, males were found to be larger and more variable in size than females in both parental and offspring generations (Table 1). Such differences are predicted consequences of sexual selection, and evidence from many species^{17,18} has shown empirically that coefficients of variation in excess of about 6% are typical of sexually selected characters.

The heritability of adult size was determined from parent-offspring regression. Regression of sons on fathers, sons on mothers, daughters on fathers and daughters on mothers yielded four coefficients, each providing an estimate of half the heritability¹⁹. The three estimates involving females (Table 2) are typical of heritabilities for many other characters¹⁹. However, the heritability between fathers and sons (70.4%) is significantly higher than any involving females. The coefficients of additive genetic variation, considered by many to provide more precise estimates of genetic variability²⁰, were also strikingly different (for males $CV_A = 9.81$, and for females $CV_A = 2.16$). The amount of genetic variability in male size is typical of fitness characters, whereas the variability in female size is typical of characters not closely associated with fitness^{20,21}. These results demonstrate that abundant additive genetic variance exists in a preferred trait in spite of strong directional, sexual selection.

TABLE 1 Mean sizes (mm) and coefficients of variation

	Sample size	Mean size (s.e.)	Coefficient of variation
Fathers	136	5.78 (0.054)	10.8
Mothers	136	4.95 (0.016)	3.7
Sons	647	5.68 (0.026)	11.7
Daughters	537	5.00 (0.011)	5.1

Animals were collected as larvae from a natural population at Träslövsläge on the west coast of Sweden. Eclosing adults were placed in a cage containing minced seaweed (*Fucus serratus*) and two days after eggs were laid, groups of 10 first-instar larvae were transferred to pots containing 50 g of minced seaweed. Previous experiments had shown that this amount of food was sufficient to prevent larval competition; overall, 87% of larvae survived to adulthood. The adults eclosing from these pots ('parental adults') were collected as virgins, and males and females from different pots set up in pairs. Again 10 first-instar larvae were transferred to new pots containing an excess of food and all F_1 progeny adults collected. A total of 136 crosses were done. The sizes of parental adults and their F_1 adult progeny were estimated by measuring wing length¹⁴. The comparison of mean wing lengths takes into account the differences in variances using the method of ref. 25. Comparing means of fathers and mothers, $t = 14.7$, d.f. = 159, $P < 0.001$. Comparing means of sons and daughters, $t = 24.1$, d.f. = 865, $P < 0.001$. Comparing variances of fathers and mothers, $F = 11.8$, $P < 0.001$. Comparing variances of sons and daughters, $F = 6.8$, $P < 0.001$.

Furthermore, the expression of the relevant loci has become uncoupled in the two sexes, such that there is much lower genetic variance of body size in females. Because this uncoupling is predicted to be an exceedingly slow process¹², it would appear that male size may have been subject to sexual selection for a considerable time.

The crosses also provide estimates of how much of the variance in offspring size is explained by the $\alpha\beta$ inversion system (Table 3). A much tighter relationship between genotype and phenotype was seen in males than in females. The correspondence of the estimates in males (76–81%) to the father-son heritability (which is the proportion of the total variance attributable to additive genetic variance¹⁹), suggests this inversion system contributes virtually all the additive genetic variance in male size. When the effect of offspring genotype on offspring phenotype was first removed (in a stepwise analysis of variance), parental size then explained very little (0.7% or less, see Table 3) of the remaining variance. This confirms that the genetic variance in the preferred character is almost entirely attributable to the $\alpha\beta$ inversion.

Male size could only be used by females as a reliable indicator of fitness in natural populations if the genetic component of variance is not swamped by the variance arising from environmental factors. Three samples were collected at roughly nine-month intervals from the population at Träslövsläge (Sweden), the sizes and karyotypes of adults determined, and the contribution of the karyotype to total variance calculated. As expected, the $\alpha\beta$ inversion accounted for a smaller proportion of the variance in size than was found in laboratory culture (the values for the three samples were: 19.5%, 17.2% and 32.3%), but it still explained, on average, almost 25%. An indicator that is 25% reliable is still likely to be more advantageous than no indicator at all.

If the loci determining adult size in seaweed flies are randomly distributed around the genome, we should expect about 10% of them to reside within the $\alpha\beta$ inversion on chromosome 1, because roughly 10% of all polytene bands are located in this region^{22,23}. Yet it appears that virtually all the relevant loci cosegregate with the $\alpha\beta$ inversion which, notably, also cosegre-

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TABLE 2 Heritabilities (%) of adult size

	Father	Mother
Son	70.4 (7.6)	49.5 (9.1)
Daughter	24.5 (7.5)	17.9 (8.1)

Standard errors of the heritability estimates are given in parentheses. Crosses were done in four batches and the means and variances differed slightly between batches; before computing regression coefficients batch effects were removed from the analysis. Heritability estimates were modified to take into account the difference in variances of males and females¹⁹. The heritability between fathers and sons was significantly higher than any of the other three values (comparing regression coefficients: normal deviate (d) > 1.76, $P = 0.019$).

TABLE 3 Percentage of variance in adult size attributable to the $\alpha\beta$ inversion system

Percentage of:	
Fathers' variance explained by fathers' karyotype	80.3
Mothers' variance explained by mothers' karyotype	10.0
Sons' variance explained by sons' karyotype	76.7
Daughters' variance explained by daughters' karyotype	6.1
Sons' variance explained by fathers' size after removing the effect of sons' karyotype	0.1
Sons' variance explained by mothers' size after removing the effect of sons' karyotype	0.5
Daughters' variance explained by fathers' size after removing the effect of daughters' karyotype	0.7
Daughters' variance explained by mothers' size after removing the effect of daughters' karyotype	0.4

The inversion karyotype of every individual was inferred from its genotype at the alcohol dehydrogenase locus using starch gel electrophoresis^{21,26}. The per cent variance values derive from sums of squares in analyses of variance after batch effects (see legend to Table 2) had been removed.

gates with variation in female preference²⁴. One possibility is that sexual selection has favoured translocation of genetic material into this inversion but, perhaps more plausibly, the loci located in or near the inversion may be the only ones to have remained polymorphic. The strong heterosis associated with the $\alpha\beta$ inversion clearly contributes to the maintenance of variation, whereas other loci may have become fixed as a result of directional selection. Sexual selection has also led to a change in the expression of the genes determining size. Either unlinked genes have become suppressed in males, or the expression of loci linked to the $\alpha\beta$ inversion has become exaggerated to the extent that other genes appear to have negligible effect. The exceptionally large variance in male size compared with female size is consistent with this interpretation. Whatever the evolutionary mechanisms involved, sexual selection has resulted in an apparent focusing of the additive genetic variance in the preferred trait into a small part of the genome, the $\alpha\beta$ inversion.

It may not be coincidence that this inversion is a major determinant of fitness^{9,10}. Because it is virtually the sole genetic determinant of variation in male size, use of the size of potential mates provides females with the opportunity to predict progeny fitness.

Whether the reliability of size as an indicator of fitness is sufficient in natural populations remains to be demonstrated. Nevertheless, all the prerequisites for female mate choice to have evolved in response to indirect selection, either by a good genes mechanism or the fisherian process, appear to exist. □

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Inhibition of skin development by targeted expression of a dominant-negative retinoic acid receptor

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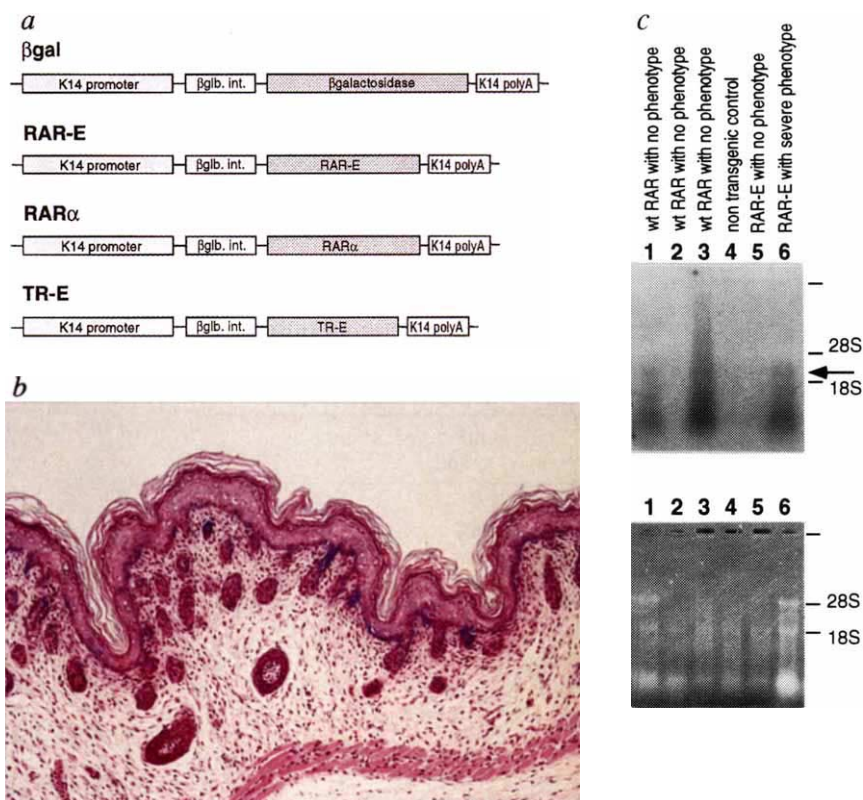
ALTHOUGH pharmacological doses of retinoic acid (RA) have a wide variety of actions *in vivo*¹, experimental difficulties have prevented a definitive assignment of its physiological functions. We recently made a dominant-negative retinoic acid receptor (RAR) by a single amino-acid substitution² which creates a dominant-negative thyroid hormone receptor³. The mutated RAR efficiently inhibited the endogenous activities of RARs (α , β , γ)². Thus, targeted expression of the mutated receptor should reveal RA functions during organogenesis by blocking RA signalling in the tissues concerned. To address this possibility, we expressed the dominant-negative RAR in the epidermis, a potential target organ of RA⁴. We report here that the resultant transgenic mice exhibited dramatic suppression of epidermal maturation, demonstrating the requirement of RA in normal skin development.

To unravel the physiological functions of RA, some investigators have disrupted the RAR genes^{5–7}. Instead, we chose the targeted expression of a dominant-negative RAR (RAR-E)². Figure 1a shows the structures of the transgenes. We first expressed the β -galactosidase gene under the keratin 14 promoter. As reported^{8,9}, the transgenic mice demonstrated clear X-gal staining in the basal cell layer of the epidermis (Fig. 1b). No other tissues examined were clearly stained (not shown).

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FIG. 1 Structure of each transgene and its expression pattern. **a**, Schematic structure of each transgene is presented. All transgenes are under the control of the human K14 promoter^{8,9} and include a rabbit β -globin intron (β gln. int.)¹⁸ and the K14 polyadenylation site^{8,9}. Each transgene contains the β -galactosidase gene, a dominant-negative RAR (RAR-E) cDNA², the parental wild-type RAR- α cDNA, or an equivalent dominant-negative TR (TR-E) cDNA^{2,3}. Unshaded and shaded boxes represent gene or cDNA fragments common and specific in each transgene, respectively. **b**, The expression pattern of the β -galactosidase transgene highlighted by the X-gal staining. The skin section of a mouse with the β -galactosidase transgene was doubly stained by X-gal and haematoxylin–eosin. The skin showed the normal phenotype. The basal cell layer and cells surrounding some hair follicles were exclusively stained by X-gal (blue). **c**, Northern blot analysis on skin RNAs (upper) and the EtBr staining pattern of the RNAs (lower). Two of three samples from wild-type RAR mice (lanes 1 and 3) and one sample from RAR-E mice with a severely affected phenotype (lane 6) demonstrated comparable RNA levels. Although the expressed RNAs were smeared, apparent signals with the expected size of about 2.4 kb were observed (indicated by an arrow). The transgenes were totally silent in the RAR-E mouse with normal phenotype (lane 5) as well as in a wild-type RAR mouse (lane 2), although both mice contained a high copy number of the transgenes (see Table 1 and its legend). The endogenous RAR- α was below detection level in all samples including the non-transgenic control (lane 4).

METHODS. The fertilized eggs from BDF1 female mice were microinjected by standard procedures¹⁹. To avoid potential cannibalization by the mothers⁶, we performed caesarean operations at E19.5 and



examined the macro- and micro-phenotypic changes. The X-gal staining was as described previously²⁰. The northern blot was probed with a human RAR- α cDNA as described previously^{21,22}.

Generally, higher staining was observed in the mice with more copies of the transgene. However, some mice, even those with high copy numbers, failed to show any X-gal staining, which might be due to the inactivation by the integrated locus.

We next introduced the dominant-negative RAR (RAR-E) as well as the wild-type RAR- α and a dominant-negative thyroid hormone receptor (TR) (referred to as TR-E, see Fig. 1a and legend)^{2,3}. The results are summarized in Table 1. The three constructions, RAR-E, RAR- α and TR-E, all produced several transgenic mice with similar efficiencies, thus excluding the possibility of the potential toxicity of a specific construction. Among the transgenic mice, eight offspring showed dramatic suppression of epidermal development, exclusively generated by the RAR-E transgene (Table 1). Although the wild-type RAR transgene demonstrated RNA levels comparable to or higher than those of the RAR-E (Fig. 1c), wild-type RAR mice did not show any phenotypic changes. This result precludes the possibility that overexpression of the mutated RAR makes the skin hypersensitive to RA and causes the phenotypic changes observed. More importantly, our results demonstrate that the phenotypic changes are completely dependent on the dominant-negative RAR. Thus, we concluded that RAR-mediated pathways, and in fact RA, are absolutely necessary in normal skin development.

Figure 2a demonstrates macro-phenotypic changes of a severely affected animal. The affected skin was tympanic and completely smooth. Moreover, neither wrinkles nor hair could be observed, although the animal had relatively normal whiskers. The affected skin seemed very thin and dried rapidly, and the animal died within a few hours. After caesarean birth, even the most severely affected animal could move and start breathing, which suggests that the other organs developed quite normally. This idea is supported by the observation that no clear macro-

phenotypic changes were evident in organs that we examined (data not shown).

Figure 2b shows the skin sections with haematoxylin–eosin staining of the affected animal and that of the control. The epidermis was severely affected and the thickness of the affected epidermis became one-fifth of that of the control. The formation of the horny and spinous layers, both of which are markers of the matured epidermis¹⁰, was greatly hindered. Furthermore, both the dermis–epidermis junction and the skin surface were completely flat, leading to loss of papillar structures and primary relief of wrinkles, respectively. Notably, wrinkles and the spinous layer both become apparent after E15.5 in the normal back-skin epidermis^{9–11}. The embryonic age of the affected epidermis may be earlier than E15.5, because the K14 promoters are active as early as E12.5 in backskin⁹. These results are surprising, because it has previously been shown that vitamin A depletion in cultured keratinocytes enhances their terminal differentiation¹² and that vitamin A deficiency in adult rodents generally induces hyperkeratosis¹³. These variances in turn suggest totally different functions of retinoic acid either *in vitro* or *in vivo*, and likewise either during or after epidermal development.

We next analysed the expression of filaggrin, a marker for the embryonically matured skin. Filaggrin is well expressed in the granular cells of only the matured skin at birth¹⁴. As reported, normal skin demonstrated the expected expression of filaggrin in the granular cells (Fig. 2c, left). In contrast, the filaggrin expression was severely decreased in the affected skin (Fig. 2c, right), again indicating immature skin development.

We next analysed the expression of keratins, differentiation markers of the keratinocytes. We observed ectopic expression of K6 and K16 in the affected skin (Fig. 3a). K6 and K16 have been observed in some pathological cases¹⁵, such as psoriasis

TABLE 1 Summary of the phenotypic changes in the transgenic mice

	Copy number			Phenotypes			No. of examined offspring
	<10	10–20	>20	Normal	Affected		
					Moderate	Severe	
RAR-E	4	4	5	5	4	4	78
RAR- α	4	2	3	9	0	0	82
TR-E	1	3	2	6	0	0	56

The numbers of identified transgenic mice are shown. The total numbers of offspring examined from each transgene are shown on the right. The copy number of each transgene was estimated by densitometric scanning of the autoradiographs from Southern or DNA dot blottings²⁹. When the average thickness of the epidermis was between 30 to 70% and less than 30% of that of the non-transgenic animal, we estimated the animals to be moderately and severely affected, respectively. The phenotypic changes were observed only from the offspring with RAR-E transgene. All 4 offspring with 10 to 20 copies of the RAR-E transgene demonstrated moderately affected phenotypes. From 5 offspring with more than 20 copies of the RAR-E transgene, 4 exhibited severely affected phenotypes and 1 had no apparent phenotype because of the inactivation of the transgene (Fig. 1c). No clear phenotype was observed from the RAR-E transgenic animals with less than 10 copies of the transgene. These results are consistent with the observation from our β -gal transgenic mice, thus indicating that the strength of the phenotypic changes are correlated with the protein levels of the RAR-E.

and in cultured keratinocytes, and these keratins have been reported to be repressed by the administration of RA¹⁶. These results are consistent with the idea that the dominant-negative RAR can block the RAR-mediated signalling pathways *in vivo*.

We also examined the expression patterns of epidermal keratins by immunohistochemical studies. The ectopic expression of both K6 and K16 was confirmed in whole spinous layers only

in the affected skin (Fig. 3b, c and legend). Furthermore, we identified differing expression of other keratins between normal and affected skins. In normal skin, anti-K10 stained both the spinous and the granular layers but not the basal cell layer (Fig. 3d) and, in contrast, anti-K5 displayed the strongest staining in the basal cell layer (Fig. 3e). The movement of expression of K5 and K14 to K1 and K10 in epidermal cells from the basal into the spinous layers is one of the first biochemical signs that a

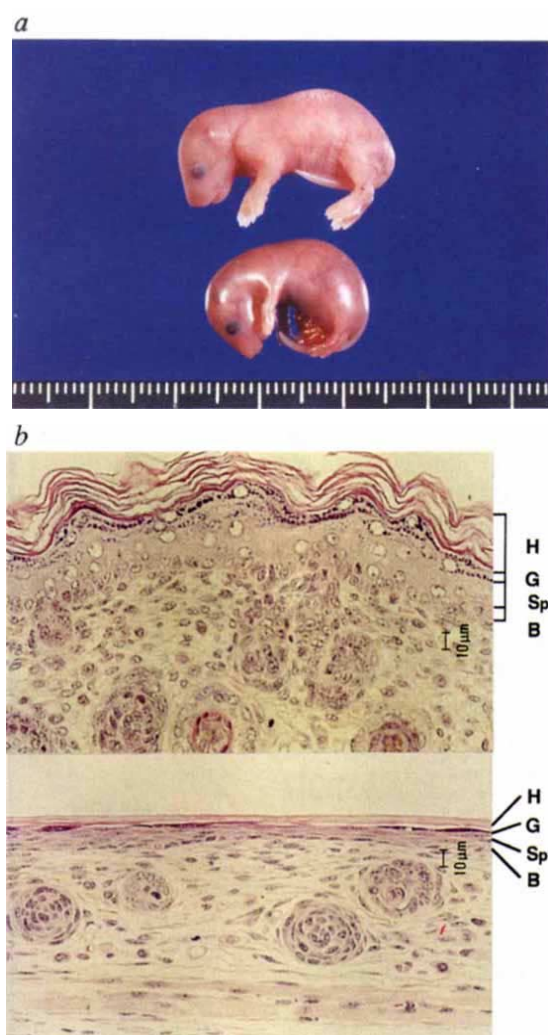


FIG. 2 Macro- and micro-phenotypic changes of the transgenic mice with a dominant-negative retinoic acid receptor. *a*, E19.5 embryos with normal phenotype (upper) and severely affected phenotype (lower). The affected skin was tympanic and completely smooth, with no wrinkles or hair. The affected skin was very fragile and the abdomen ruptured when the umbilical cord was disconnected. Scale, each mark represents 1 mm. *b*, The skin sections are presented from the mice shown in *a*, with haematoxylin-eosin staining. The thickness of affected epidermis (lower) became less than one fifth of that of the control (upper). The horny layer, spinous layer and granular layer were all much less prominent. In the affected skin, not even hair canals were formed within the hair follicles. This is also characteristic of embryonically immature skin (before E15.5)²³ and thus there were no open hair follicles. Scale bars, 10 μ m. H, Horny layer; G, granular layer; Sp, spinous layer; B, basal cell layer. *c*, Skin sections from the mice shown in *a* were stained by anti-filaggrin antibody. Normal skin demonstrated clear expression of filaggrin in the granular cells (left); in contrast, the filaggrin expression was severely depressed in the affected skin (right). The fixed skin sections were treated by the primary monoclonal anti-filaggrin antibody, BT-576 (BTI, USA), followed by the procedures described in Fig. 3 legend. G, granular layer.

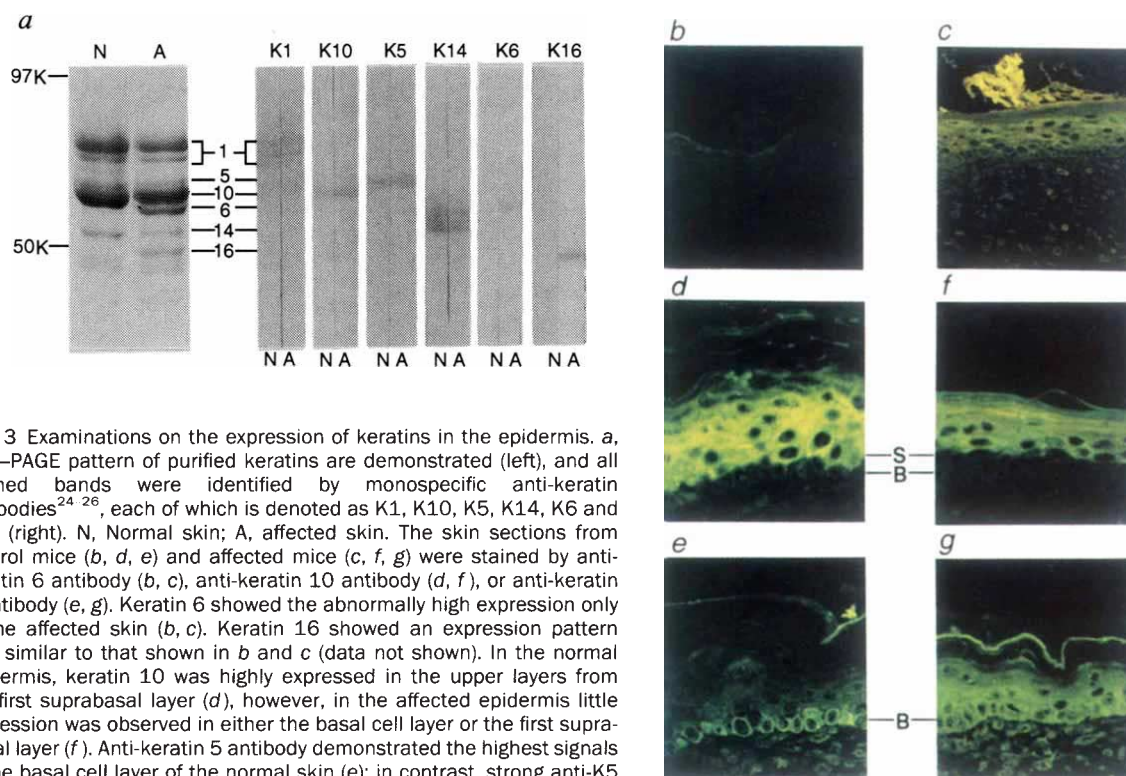


FIG. 3 Examinations on the expression of keratins in the epidermis. *a*, SDS-PAGE pattern of purified keratins are demonstrated (left), and all stained bands were identified by monoclonal anti-keratin antibodies^{24, 26}, each of which is denoted as K1, K10, K5, K14, K6 and K16 (right). N, Normal skin; A, affected skin. The skin sections from control mice (*b*, *d*, *e*) and affected mice (*c*, *f*, *g*) were stained by anti-keratin 6 antibody (*b*, *c*), anti-keratin 10 antibody (*d*, *f*), or anti-keratin 5 antibody (*e*, *g*). Keratin 6 showed the abnormally high expression only in the affected skin (*b*, *c*). Keratin 16 showed an expression pattern very similar to that shown in *b* and *c* (data not shown). In the normal epidermis, keratin 10 was highly expressed in the upper layers from the first suprabasal layer (*d*), however, in the affected epidermis little expression was observed in either the basal cell layer or the first suprabasal layer (*f*). Anti-keratin 5 antibody demonstrated the highest signals in the basal cell layer of the normal skin (*e*); in contrast, strong anti-K5 staining extended into the spinous cell layers in the affected skin (*g*). The comparable expression levels of K5 in both skins (*a*) might be explained as follows; K5 is present suprabasally in the normal skin but its epitope is masked if the normal mouse skin is similar to human. S, suprabasal layer; B, basal cell layer.

METHODS. Epidermal keratins were purified as described²⁷, resolved by 12% SDS-PAGE, and stained with Coomassie brilliant blue. Western blots were as described²⁸ and the signals were detected by the alkaline phosphatase colour detection system (Promega, USA). The fixed skin

sections were treated by the primary monoclonal antibody of CK10 (DAKO, Denmark) or polyclonal rabbit anti-keratin 6 or guinea-pig anti-keratin 5 antibody, then washed with PBS and incubated with the secondary antibody (FITC-labelled antibodies (DAKO, Denmark)). Preparations were examined and photographed on an immunofluorescence microscope.

keratinocyte has undergone a commitment to differentiate terminally¹⁷. In contrast, the expression of K10 in affected skin was excluded not only from the basal cell layer but also from cells of one layer above the basal cell layer (Fig. 3*f*). Furthermore, strong anti-K5 staining extended into the spinous cell layers (Fig. 3*g*). The expression patterns of K1 and K14 paralleled those of K10 and K5, respectively, in both normal and affected skins (data not shown). Collectively, these data suggest that the biochemical programme of differentiation in the RAR-E mice is aberrant, and that the induction of the switch in keratin expres-

sion is delayed. From these results, we propose that the blockage of RAR-mediated signalling at the basal cell layer results in an inhibition of keratinocyte differentiation, thus leading to immature epidermis.

Here we have presented a new strategy for examining the physiological functions of RA in a tissue-specific manner. This method has revealed the absolute requirement of RA for normal skin development and is theoretically applicable to any organ, and thus may open a way to examine the physiological roles of RA during embryogenesis as well as in adults. □

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Protection against Fas-dependent Th1-mediated apoptosis by antigen receptor engagement in B cells

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CYTOTOXIC CD4⁺ Th1-cells induce cell death by triggering a Fas-dependent apoptotic pathway¹⁻⁶. Potential targets include activated B cells^{3,7}, but it is not known whether the mode of B-cell stimulation influences susceptibility to Th1-mediated cytotoxicity. Here we report that CD40-ligand-stimulated B cells were

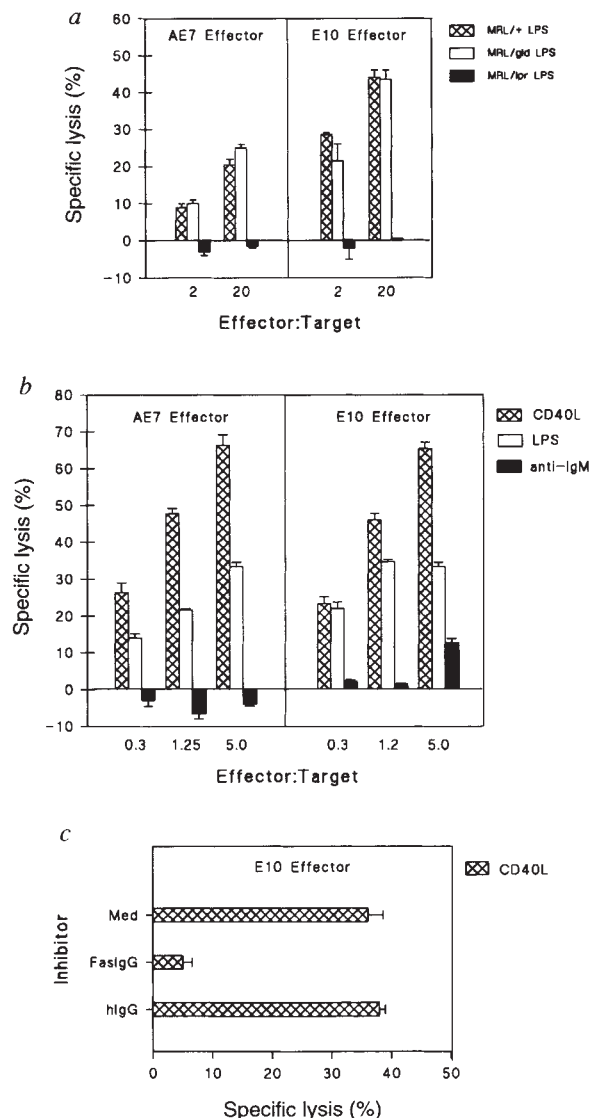
extremely sensitive, whereas anti-IgM-stimulated B cells were resistant, to Fas-mediated apoptosis. B cells stimulated by both CD40L and anti-IgM were not susceptible to cytotoxicity, demonstrating that anti-IgM-mediated protection is an active, dominant process. Resistance to Th1-mediated cytotoxicity was similarly observed in CD40L-stimulated 3-83 (anti-H-2K^b)⁸ transgenic B cells co-cultured with H-2K^k or H-2K^b (but not H-2K^d) splenocytes. These results indicate that B cells can participate in regulating their own destruction. Protection against Fas-dependent apoptosis afforded by immunoglobulin-receptor engagement may constitute a fail-safe mechanism that eliminates bystander B cells activated by CD40L-expressing T cells, but ensures survival of antigen-specific B cells.

To confirm that target-cell lysis occurred through a Fas-dependent mechanism in these assays, primary B cells were stimulated and used as targets in a CD4⁺ Th1-cell-mediated cytotoxicity (Th1-CMC) assay, as described⁹. Lipopolysaccharide (LPS)-stimulated B cells obtained from Fas-expressing MRL/+ and MRL/*gld* (Fas ligand-deficient^{10,11}) mice were susceptible to lysis, whereas B cells from Fas-deficient¹² MRL/*lpr* mice were not (Fig. 1a).

Additional forms of stimulation were then tested for induction of susceptibility to Th1-CMC. Normal B cells stimulated by CD40-ligand (CD40L) were readily lysed by Th1-effector populations, with greater efficiency than LPS-stimulated target cells (Fig. 1b). Lysis of CD40L-stimulated B-cell targets was com-

FIG. 1 Susceptibility of stimulated B cells to Fas-dependent, Th1-mediated cytotoxicity. Primary B cells were stimulated *in vitro* for 2 days, washed, labelled with ⁵¹Cr, and assessed as targets in cytotoxic assays using Th1 effector populations. **a**, B cells obtained from mice that do (MRL/+ and MRL/*gld*) and do not (MRL/*lpr*) express Fas were stimulated with LPS before assay for susceptibility to Th1-cell-mediated cytotoxicity (Th1-CMC) using the CD4⁺ Th1-cell lines AE7 and E10 at the effector:target cell ratios shown. **b**, B cells obtained from BALB/c mice were stimulated with either LPS, supernatant containing a chimaeric CD40L-CD8 α fusion protein in the presence of crosslinking anti-CD8 antibody (CD40L), or F(ab')₂ fragments of goat anti-mouse IgM antibody (anti-IgM), as indicated, before serving as target populations in a Th1-CMC assay using the CD4⁺ Th1-cell lines AE7 and E10 at the effector:target cell ratios shown. **c**, B cells obtained from BALB/c mice were stimulated with CD40L and used as targets for E10 Th1-effector cells at an effector:target cell ratio of 4:1 (Med) or under the same conditions in the added presence of soluble chimaeric Fas-human IgG fusion protein (Fas-IgG), or control human IgG (hIgG).

METHODS. B cells were prepared from murine splenocytes by depletion of T cells with anti-Thy 1.2, anti-CD4 and anti-CD8 plus rabbit complement, and depletion of macrophages by plate adherence. B cells at $1.2\text{--}1.6 \times 10^6$ per ml were stimulated for 2 days with either LPS, CD40L or anti-IgM. LPS from *Salmonella typhimurium* was used at $25 \mu\text{g ml}^{-1}$. Supernatant from transfected J558L cells that secrete a chimaeric CD40L-CD8 α fusion protein¹³ was collected and dialysed against 25,000 molecular mass cutoff dialysis tubing¹⁵; this supernatant was used at a 1:8 dilution, along with similarly dialysed supernatant containing anti-CD8 antibody derived from the 53-6-72 hybridoma. Optimal dilutions of chimaeric CD40L and anti-CD8 were determined on the basis of B-cell staining and induction of B-cell proliferation. Polyclonal goat anti-mouse IgM antibody (F(ab')₂ fragments) was used at $5\text{--}7.5 \mu\text{g ml}^{-1}$. Following stimulation, cells were washed and viable cells recovered by density gradient centrifugation. These cells were labelled with ⁵¹Cr for 60 min, washed, and then used as targets in 4-h lectin-dependent cytotoxicity assays with AE7 and E10 CD4⁺ Th1-cell lines as previously described³. Fas-IgG was obtained from NIH 3T3 cells transfected with a construct consisting of RT-PCR-amplified Fas extracellular domain, including the leader sequence, cloned into a human IgG1 constant region expression vector; the fusion protein was purified from the serum of SCID mice previously inoculated subcutaneously with the Fas-IgG-3T3 transfectant using protein A affinity chromatography. Fas-IgG and control human IgG were used at $10 \mu\text{g ml}^{-1}$. Triplicate wells ($2\text{--}4 \times 10^4$ target cells per well) were set up to assay spontaneous release (no effector cells), experimental release (with Th1-effector cells) and total release (determined by lysing target cells with 0.5% NP-40). Per cent specific lysis was calculated by the formula: (experimental release - spontaneous release)/(total release - spontaneous release) $\times 100$. Spontaneous release was in all groups less than 30% of total releasable counts.



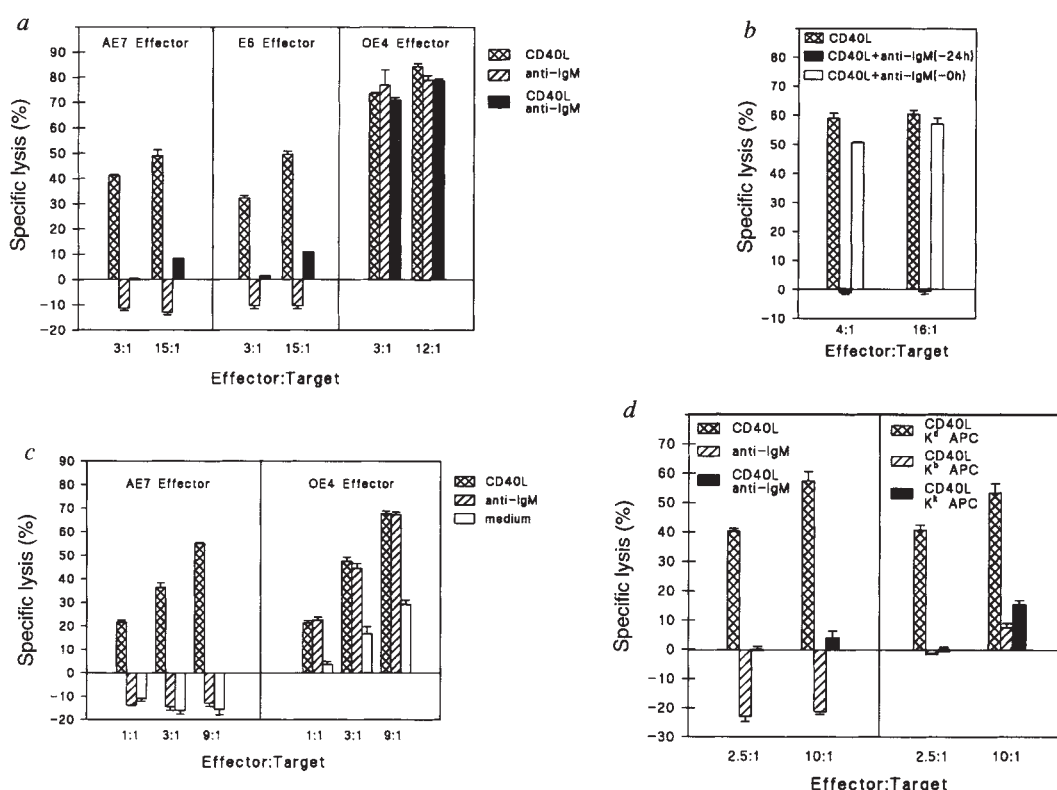
pletely blocked by addition of soluble Fas-IgG, but not human IgG (hIgG), to the cytotoxicity assay (Fig. 1c), demonstrating dependence on Fas expression. In contrast, anti-IgM-stimulated B cells were not lysed under these conditions (Fig. 1b). These results demonstrate marked dissimilarity in susceptibility to Th1-CMC between B cells activated by two distinct modes of stimulation, each of which is individually capable of inducing cell-cycle progression^{13,15}.

It was then of interest to determine whether the failure of anti-IgM-stimulated B cells to be lysed by Th1-effector cells represents a passive process, in which signalling is simply deficient for this outcome, or an active, dominant process, in which cytotoxicity is suppressed. To evaluate these alternatives, B cells were stimulated simultaneously with CD40L plus anti-IgM (CD40L/anti-IgM), and then tested as targets. CD40L/anti-IgM-stimulated B cells were not lysed by Th1-effector populations, and thus the susceptibility to cytotoxicity typically imparted by CD40L was suppressed by concurrent anti-IgM treatment (Fig. 2a). This resistance is unlikely to reflect a direct blocking effect of the anti-IgM antibody, because B cells were washed free of anti-IgM hours before the Th1-CMC assay and would by then no longer

contain substantial amounts of membrane-bound exogenous immunoglobulin¹⁶. Moreover, in a direct test of potential blocking, anti-IgM added to CD40L-stimulated B cells at the start of the assay did not alter specific cell lysis in contrast to anti-IgM present during the last 24 h of culture (Fig. 2b).

Protection against Th1-CMC cannot be attributed to diminished activation of B cells stimulated through both receptors, as S-phase entry of CD40L/anti-IgM-stimulated B cells was greater than that of B cells stimulated by CD40L alone (Fig. 2 legend). Further, the absence of Th1-CMC toward CD40L/anti-IgM-stimulated B cells cannot be attributed to insufficient Fas expression. Quantitative reverse transcribed-polymerase chain reaction (RT-PCR) showed comparable levels of *Fas* gene expression in CD40L/anti-IgM-stimulated B cells, as compared to B cells stimulated by CD40L alone (Fig. 3a). Evaluation of Fas by surface staining¹⁷ similarly revealed virtually identical levels of expression in these two B-cell populations (Fig. 3b). These data further demonstrate that anti-IgM exposure does not block all effects of CD40L stimulation. Of note, anti-IgM-stimulated B cells expressed levels of Fas that, although somewhat elevated in comparison to unstimulated B-cells, fell short of the levels of

FIG. 2 Resistance of B cells stimulated by the combination of CD40L plus anti-IgM to Fas-dependent, Th1-mediated cytotoxicity. a, Primary B cells were stimulated with either CD40L, anti-IgM, or the combination of CD40L plus anti-IgM, at the same final concentrations as given in the legend to Fig. 1, for 2 days, as indicated. B-cell sensitivity to Fas-dependent cytotoxicity was assessed using the CD4⁺ Th1-cell lines AE7 and E6. B-cell susceptibility to non-Fas-dependent, CD8⁺ T-cell-mediated cytotoxicity was assessed in the presence of Ca²⁺ using the OE4 T-cell line. b, B cells were stimulated with CD40L for 2 days either alone or in the added presence of anti-IgM antibody during the last 24 h of culture (–24 h), after which B-cell susceptibility to Fas-dependent cytotoxicity was assessed using the CD4⁺ Th1-cell line AE7 at the effector:target cell ratios shown. The assay of CD40L-stimulated B cells was done in duplicate; in one set, anti-IgM was added just before the addition of AE7 effector cells (–0 h). c, CD40L-stimulated, anti-IgM-stimulated, and unstimulated (medium) B cells were assessed for sensitivity to Fas-dependent cytotoxicity using the CD4⁺ Th1-cell line AE7, and for sensitivity to non-Fas-dependent, CD8⁺ T-cell-mediated cytotoxicity using the OE4 T-cell line. d, Primary 3-83 B cells were stimulated with either CD40L, anti-IgM, or the combination of CD40L plus anti-IgM, for 2 days, as described above. In addition, 3-83 B cells were stimulated with CD40L for 2 days in the presence of an equal number of antigen-presenting cells (APC) expressing either K^d, K^b or K^k. Stimulated B cells were tested for susceptibility to Fas-dependent cytotoxicity using the AE7 CD4⁺ Th1-cell line at the effector:target cell ratios indicated. One of two comparable experiments is shown.

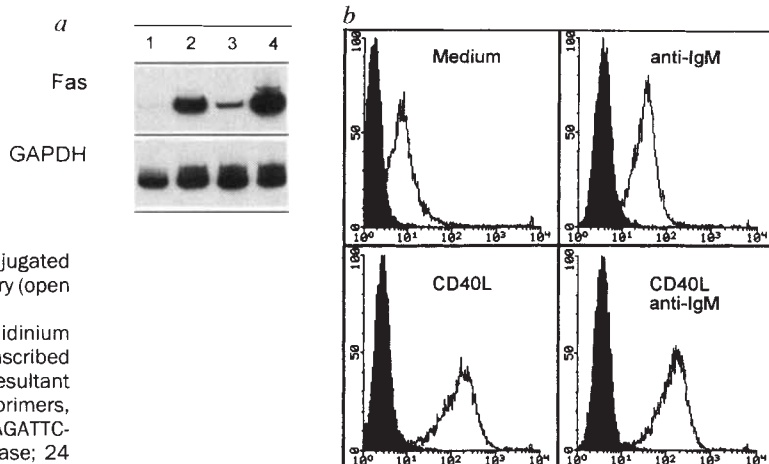


–0 h). c, CD40L-stimulated, anti-IgM-stimulated, and unstimulated (medium) B cells were assessed for sensitivity to Fas-dependent cytotoxicity using the CD4⁺ Th1-cell line AE7, and for sensitivity to non-Fas-dependent, CD8⁺ T-cell-mediated cytotoxicity using the OE4 T-cell line. d, Primary 3-83 B cells were stimulated with either CD40L, anti-IgM, or the combination of CD40L plus anti-IgM, for 2 days, as described above. In addition, 3-83 B cells were stimulated with CD40L for 2 days in the presence of an equal number of antigen-presenting cells (APC) expressing either K^d, K^b or K^k. Stimulated B cells were tested for susceptibility to Fas-dependent cytotoxicity using the AE7 CD4⁺ Th1-cell line at the effector:target cell ratios indicated. One of two comparable experiments is shown.

METHODS. B cells were prepared from murine splenocytes and then stimulated, labelled with ⁵¹Cr, and used as targets in lectin-dependent cytotoxicity assays as described in the legend to Fig. 1. a, B cells were exposed to CD40L, anti-IgM, and the combination of CD40L plus anti-IgM, as indicated, for 2 days. B cells were washed, and, to assess proliferative activity, aliquots of these cells (1×10^5) were placed in microtitre wells and incorporation of [³H]thymidine (20 Ci mmol^{-1} ,

$0.5 \mu\text{Ci per well}$) during a 6 h labelling period was assessed. Mean ($\pm$ s.e.m.) values of triplicate wells for one (typical) experiment were as follows ($\text{c.p.m.} \times 10^{-3}$): CD40L, 64.9 ± 0.6 ; anti-IgM, 123.6 ± 5.8 ; CD40L plus anti-IgM, 149.1 ± 9.1 . B-cell susceptibility to Fas-dependent (AE7, E6) and non-Fas-dependent (OE4) T-cell-mediated cytotoxicity was assessed, the latter specifically in the presence of Ca²⁺. b, B cells were stimulated with CD40L with or without anti-IgM during the last 24 h of culture (–24 h) and tested as targets for Th1-CMC using AE7 effector cells, as described above. B cells treated with CD40L alone were assayed in duplicate; in one set anti-IgM at $10 \mu\text{g ml}^{-1}$ was added just before the addition of effector cells (–0 h). c, Unstimulated B cells (medium) and B cells treated for 2 days with either CD40L or anti-IgM, as indicated, were used as targets in cytotoxicity assays with AE7 and OE4 effector cells, as described in a. d, Transgenic B cells ($2 \times 10^6 \text{ ml}^{-1}$) were cocultured with CD40L plus an equal number of T-cell depleted, mitomycin-C-treated spleen cells, obtained from either BALB/c (K^d), B6 (K^b) or MRL/+ (K^k) mice. B cells were used as targets for Th1-mediated cytotoxicity as described above.

FIG. 3 Expression of Fas mRNA and protein in stimulated B cells. Primary B cells were assayed directly (medium) or stimulated with either CD40L, anti-IgM, or the combination of CD40L plus anti-IgM, for 2 days, as indicated, and assayed. **a**, Quantitative RT-PCR for Fas and for GAPDH was done on RNA isolated from stimulated B cells; incorporation of [³²P]CTP into oligonucleotide product was assessed by autoradiography. Lane 1, untreated B-cells; lane 2, B cells stimulated with CD40L; lane 3, B cells stimulated with anti-Ig; lane 4, B cells stimulated with the combination of CD40L plus anti-Ig. **b**, Stimulated B cells were stained with phycoerythrin-conjugated Jo-2 anti-Fas monoclonal antibody and analysed by flow cytometry (open curves); control samples were unstained (shaded curves). **METHODS.** **a**, RNA was obtained from B cells using a guanidinium method (RNAzol, Tel-Test, Friendswood, TX) and was reverse-transcribed with Moloney murine leukaemia virus reverse transcriptase. Resultant cDNA served as a template for PCR amplification using the primers, ATCCGAGCTCTGAGGAGGCGGGTTCATGAAAC, and GGAGGTCTAGATTC-AGGGTCATCCTG, with 1 unit *Thermus aquaticus* DNA polymerase; 24 cycles of amplification were done consisting of 94 °C for 1.5 min, 60 °C for 2 min and 72 °C for 2 min, beginning with 2 min at 94 °C and ending with 5 min at 72 °C. Buffer conditions included pH 8.0, Mg²⁺ 1.3 mM. [³²P]CTP present in the reaction mixture was incorporated into the amplified product which was separated by electrophoresis on a 4% non-denaturing polyacrylamide gel and subjected to autoradiography. Molecular mass markers were used to determine the size of the ampli-



Fas elicited by CD40L; thus, suboptimally induced Fas expression could contribute to the insensitivity of anti-IgM-stimulated B cells to Th1-CMC, although this explanation cannot be invoked for CD40L/anti-IgM-stimulated B cells.

The resistance of CD40L/anti-IgM-stimulated B cells to Th1-CMC does not represent suppression of all forms of cytotoxicity. This was demonstrated by testing B-cell targets activated in various ways for susceptibility to lysis using a CD8⁺ T-cell line, under conditions in which the perforin/granzyme pathway dominates cytotoxic activity¹⁸. B cells stimulated by CD40L, by anti-IgM, and by CD40L plus anti-IgM, were equally susceptible to CD8⁺ T-cell-mediated cytotoxicity, indicating that protection afforded by antigen-receptor engagement is specific for resistance to Fas-dependent effector mechanisms, and does not result from a global failure of conjugate formation (Fig. 2a). Unstimulated B cells were also lysed by CD8⁺ effector cells (although to a lesser extent than stimulated B cells, consistent with previous work¹⁹), but were not susceptible to Fas-dependent cytotoxicity^{20,21} (Fig. 2c).

The correspondence of anti-IgM-mediated protection against Th1-CMC to more physiological antigen-receptor engagement was tested by using 3-83 (anti-H-2K^b) B-cell receptor transgenic mice⁸. Transgenic B cells were susceptible to Th1 killing following activation with CD40L and this was reversed by concurrent anti-IgM stimulation, similar to the results obtained with normal B cells (Fig. 2d). Importantly, the sensitivity of CD40L-stimulated 3-83 B cells to Th1-mediated cytotoxicity was also reversed by coculture of these cells with K^k- or K^b-expressing T-cell-depleted and mitomycin-C-treated spleen cells, but not by coculture with syngeneic K^d cells that do not engage the 3-83 immunoglobulin receptor (Fig. 2d). In transgenic, as with normal B cells, Fas expression was comparably induced in all CD40L-stimulated groups (data not shown).

Thus, immunoglobulin receptor engagement by either anti-IgM or specific antigen blocks CD40L-induced susceptibility to Fas-dependent Th1-CMC. These results lead to the important conclusion that the B cell itself can actively participate in regulating its own susceptibility to Th1-CMC, in a receptor- and stimulus-specific fashion.

It has been reported that CD40-derived signals reverse several forms of B-cell death^{6,22-24}, including apoptosis that appears to occur endogenously as an apparently developmental event in germinal centres^{25,26}, suggesting that CD40L may act as a viability factor for B cells. However, as shown here, CD40L induces marked susceptibility to Fas-dependent Th1-mediated

apoptosis, unless sIg-mediated signals are contemporaneously delivered. These observations suggest a Faustian hypothesis for T-B interaction and resolution of the problem of nonspecifically stimulated bystander B cells. Activated T cells, through CD40L, may indeed enhance B-cell viability. However, with the same stroke these B cells become susceptible to lysis by Th1-effector cells that (we suggest) act to eliminate such stimulated bystander B cells. This fate is averted if CD40L-stimulated B-cells are concurrently signalled through sIg, thus preserving antigen-specific responses. This model for complex regulation of induced apoptosis by the target cell may have parallels in other potential target systems, such as malignantly transformed cells that express resistance to Fas-dependent apoptosis²⁷ and in which therapeutic options may be determined by susceptibility to inducible programmed cell death²⁸. □

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Fasciclin III as a synaptic target recognition molecule in *Drosophila*

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FASCICLIN III, a cell adhesion molecule of the immunoglobulin superfamily^{1–3}, is expressed by motor neuron RP3 and its synaptic targets (muscle cells 6 and 7) during embryonic neuromuscular development of *Drosophila*⁴. We report here that RP3 often incorrectly innervates neighbouring non-target muscle cells when these cells misexpress fasciclin III, but still innervates normal targets in the *fasciclin III* null mutant. Fasciclin III manipulations do not influence target selections by other motor neurons, including fasciclin III-expressing RP1. We propose that fasciclin III acts as a synaptic target recognition molecule for motor neuron RP3, and also that its absence can be compensated for by other molecule(s).

A mechanism for synaptic connectivity where each target cell bears a unique molecular label was proposed by Sperry⁵. However, testing this 'chemoaffinity theory' is difficult, because it requires both manipulating molecular expression and analysing connections with single-cell resolution.

The embryonic and larval neuromuscular system of *Drosophila melanogaster* consists of a small number of uniquely identified motor neurons and muscle cells^{4,6–10}. In late embryos (14–16 h AEL (after egg laying at 25 °C)) motor neuron RP3 innervates muscle cells 6 and 7, whereas its neighbour, RP1, targets muscle cell 13 (Fig. 1a)^{4,8}. Once they have left the central nervous system (CNS) through mechanisms independent of its target(s)^{9–13}, each RP growth cone clearly prefers its normal target(s) over the several other muscle cells within filopodial reach, and is little affected by either the deletion or addition of neighbouring non-target muscle cells^{9,10}. Such *in vivo* studies suggest that the last step in growth cone guidance must involve target-specific attractive recognition molecules.

These motor neurons and muscle cells express various combinations of cell surface molecules (such as fasciclin I¹⁴, fasciclin II¹⁵, fasciclin III^{1–4}, neuromusculin¹⁶, connectin¹⁷, Toll^{17,18}), which potentially mediate their specific interactions. Fasciclin III is a transmembrane glycoprotein with three extracellular immunoglobulin domains^{2,3}, and functions as a homophilic cell adhesion molecule *in vitro*². In late embryos, fasciclin III is expressed by both RP3 (Fig. 1c) and its targets (muscle cells 6 and 7; Fig. 1f), along with a few other neurons (such as RP1) and two oblique muscle cells (15 and 16)^{1,4}. The arrival of the fasciclin III-positive RP3 growth cone near its targets coincides with the onset of fasciclin III expression by its target muscle cells (Fig. 1b)¹⁸. Thus, fasciclin III has attributes consistent with a specific 'target recognition molecule' for motor neuron RP3.

We conducted two tests to determine whether fasciclin III is necessary and/or sufficient for RP3 target selection *in vivo*. As a control we examined the behaviour of the RP1 growth cone which, despite its expression of fasciclin III, normally innervates the fasciclin III-negative muscle cell 13.

TABLE 1 Summary of synaptic target selections by RP growth cones

	RP3			
	Wild type* (n = 15)	<i>fasciclin III</i> null (n = 17)	<i>Mhc'-fasciclin III</i> [×1]† (n = 10)	<i>Mhc'-fasciclin III</i> [×2]‡ (n = 21)
Targeted cell(s)				
Correct targets 6 and 7	15 (100%) 15	16 (94%) 16	4 (40%) 4	3 (14%) 3
Incorrect targets	0 (0%)	1 (6%)	6 (60%)	18 (86%)
14	—	—	—	5
13	—	1	1	3
14 and 30	—	—	2	2
7§	—	—	—	2
15 and 16	—	—	—	1
15	—	—	—	1
30	—	—	1	—
13, 14, and 30	—	—	1	—
13 and 14	—	—	1	—
6 and 13	—	—	—	1
6, 7, 13	—	—	—	1
6, 7, and 14	—	—	—	1
6, 7, and next segment	—	—	—	1
RP1				
	(n = 11)	(n = 9)	(n = 9)	(n = 10)
Correct target	11 (100%)	9 (100%)	9 (100%)	10 (100%)
13	11	9	9	10

Data on the basis of intracellular dye injection into individual RP neurons in wild-type, *fasciclin III* null, and *Mhc'-fasciclin III* embryos (15–16 h AEL). See Fig. 1a for muscle cell identities. Dashes, Absence of such examples.

* The newly obtained wild-type data confirmed the previously published data^{4,9}.

† Heterozygous transgenic embryos carried a single copy of the *Mhc'-fasciclin III* mini gene in the second chromosome.

‡ Homozygous transgenic embryos carried two copies of the *Mhc'-fasciclin III* mini gene on either the second (n = 16 for RP3, and 6 for RP1) or third (n = 5 for RP3, and 4 for RP1) chromosome. Both the levels of *fasciclin III* misexpression and the RP growth cones' target selections were similar in these two independent transgenic lines.

§ Muscle cell 7 was innervated from its proximal side, and not at the cleft between 6 and 7 as in the wild type.

|| Muscle cells 6 and 7 of an adjacent segment were also innervated.

Our first experiment examined the *fasciclin III* null mutant^{19,20}. The mutant is viable, with a normal embryonic nervous system^{19,20} (compare with Fig. 1d, g). Intracellular dye injection revealed normal axon pathway and target selections by both RP3 (n = 16/17) and RP1 (n = 9/9) (compare Fig. 2b, e to c, f; Table 1). Therefore, fasciclin III expression is dispensable for target selection by RP3 under otherwise normal conditions.

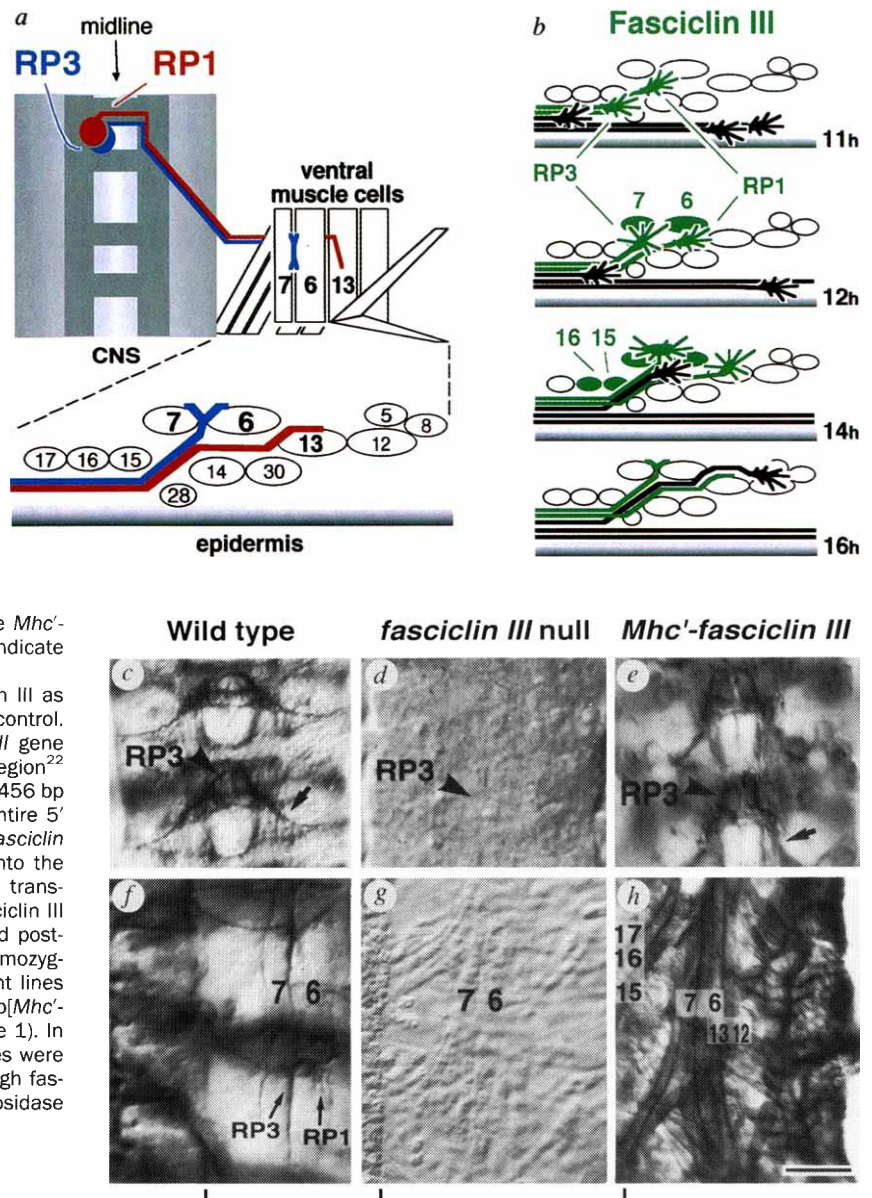
There are two possibilities to explain this observation: (1) fasciclin III may be irrelevant for RP3 target recognition, or (2) it plays a positive role but its absence can be compensated for by other mechanism(s).

Our second experiment has demonstrated that the latter is most likely. Using a muscle-specific (*Myosin heavy chain* gene) promoter^{21,22}, we generated transgenic flies which ectopically express fasciclin III in all the skeletal muscle cells (Fig. 1h). The misexpression started when RP growth cones would normally begin contacting the ventral muscle cells (11–12 h AEL) about 1 h earlier than the onset of endogenous muscle expression and continued through the larval development, beyond the time at which it is normally downregulated. In the heterozygous embryos the level of induced fasciclin III expression was about the same as that of the wild-type expression in muscle cells 6 and 7, whereas it was noticeably higher in the homozygous embryos (Fig. 1h) which showed reduced post-embryonic viability. The expression in the CNS and other tissues remained the same as in the wild type (Fig. 1e). The inappropriate expression was limited to all muscle cells, which were thus made equivalent in terms of fasciclin III expression.

Fasciclin III misexpression resulted in no gross developmental defects of the CNS, peripheral nervous system and musculature of the embryos. However, intracellular dye injection revealed a

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FIG. 1 *Drosophila* neuromuscular system. *a*, RP motor neurons and their target muscle cells. *b*, Temporal control of fasciclin III expression (from ref. 18). In the periphery, RP1 growth cone precedes RP3 growth cone by about 1 h. Although coincidental, expression in the muscle cells 6 and 7 is not induced by the arrival of the RP3 growth cone⁶. Transient fasciclin III expression by muscle cells 15 and 16 occurs after RP3 growth cone extends past them. *c–h*, Fasciclin III immunocytochemistry. The two CNS segments (*c–e*) and two right hemisegments in the musculature (*f–h*) of 12–14 h AEL embryos are shown. *c, f*, In the wild type, fasciclin III is expressed by the bilaterally paired RP3 (*c*, arrowhead) and RP1 (out of focus), their midline-crossing axons (*c*, arrow), a few other neurons along the RP axons, the 'exit' glia near the edge of the CNS, muscle cells 6 and 7 (at their 'cleft'), RP growth cones (*f*, arrows), and the muscle insertion sites of muscle cells 6 and 7^{1,4}. Photograph in *d* is from ref. 4. *d, g*, No fasciclin III is detected in the *fasciclin III* null mutant. *e, h*, In the *Mhc'-fasciclin III* transgenic embryos, the CNS expression pattern is normal whereas in the periphery all the skeletal muscle cells (including those unlabelled) express fasciclin III. The photos show the *Mhc'-fasciclin III* homozygous embryos. Short vertical bars indicate the edges of the CNS in *f–h*. Scale bar, 10 μ m. METHODS. mAb 2D5¹ was used to visualize fasciclin III as described⁴. Canton-S strain was used as the wild-type control. The *fasciclin III*^{E25} null mutant lacks the *fasciclin III* gene function^{19,20}. A mini gene containing the promoter region²² of the *Myosin heavy chain* genomic DNA (*Mhc'*; 456 bp upstream of the start of the transcription and the entire 5' untranslated region)²¹ and the coding sequence of *fasciclin III* cDNA (from +106 to +2,455 bp)² were cloned into the pCaSpeR4 vector for genomic transformation²⁹. Five transgenic lines carrying this mini gene misexpressed fasciclin III in all skeletal muscles. They exhibited much reduced post-embryonic viability when homozygosed. We used homozygous and heterozygous embryos from two independent lines (*w*; p[*Mhc'-fasciclin III*, *w*⁺]/SM5, p[*eve'-lacZ*] and *w*; p[*Mhc'-fasciclin III*, *w*⁺], *Dp/TM3*, *Sb*) for analyses (see Table 1). In those experiments where embryos of mixed genotypes were used, each embryo was tested for its genotype through fasciclin III immunocytochemistry and β -galactosidase histochemistry.



remarkable anomaly that RP3 often innervates 'incorrect' targets (for example, Fig. 2*a, d*), either in addition to or instead of the normal target muscle cells (Table 1).

As the dosage of misexpressed fasciclin III doubled, so did the frequency of RP3 misinnervating 'incorrect' targets from 60% ($n=10$) to 86% ($n=21$) (Table 1). This suggests that target selection by the RP3 growth cone is influenced not only by the spatial-temporal control of fasciclin III expression, but also by its dosage.

Incorrect innervation by RP3 occurred only on those ventral muscle cells which its growth cone filopodia would normally contact (6, 7, 13, 14, 15, 16 and 30). Not innervated by RP3 were nearby non-muscle cells (such as peripheral glia and sensory neurons) and muscle cells that lie outside the filopodial reach (12, 17, 28 and more dorsolateral ones) (Fig. 2*g*, Table 1). Therefore, the experimentally induced expression of fasciclin III is sufficient to transform the muscle cells into acceptable synaptic targets of RP3.

Misexpressed fasciclin III in the musculature does not act merely as a nonspecific 'glue' for growth cones. Even in the homozygotes where the level of misexpressed fasciclin III was much higher than normally seen on muscle cells 6 and 7, the overall neuromuscular organization appeared normal (not shown). Intracellular dye injection confirmed normal axon path-

way selection by fasciclin III-negative motor neurons (aCC and RP2, $n=3$ and 2) (not shown). Most strikingly, target selection by RP1, a fasciclin III-positive motor neuron which normally innervates fasciclin II-negative target, remained unaffected by fasciclin III misexpression ($n=9$ and 10 for hetero- and homozygotes; Fig. 2*d*, Table 1).

On the basis of these results, we propose that fasciclin III can act as a synaptic target recognition molecule during neuromuscular development in the *Drosophila* embryos. The simplest model consistent with our finding is that under normal circumstances fasciclin III and additional factor(s) both serve as specific target recognition molecules for RP3, but that their functions are largely overlapping. Considering that fasciclin III is a homophilic adhesion molecule², it is likely that fasciclin III on the RP3 growth cone directly interacts with fasciclin III on the target muscle cells.

Growth cones are known to be guided by multiple molecular cues. Diffusible molecules (such as acetylcholine²³, netrins²⁴, collapsin²⁵, nitric oxide²⁶) exert long-distance but relatively coarse influence on growth cone navigation. In contrast, cell-bound molecules (such as NCAM²⁷, fasciclin II¹⁵, connectin^{17,28}) are proposed to mediate local interactions. Some of them can act as 'repulsive target recognition molecules' (such as connectin²⁸). We have shown here evidence that fasciclin III is

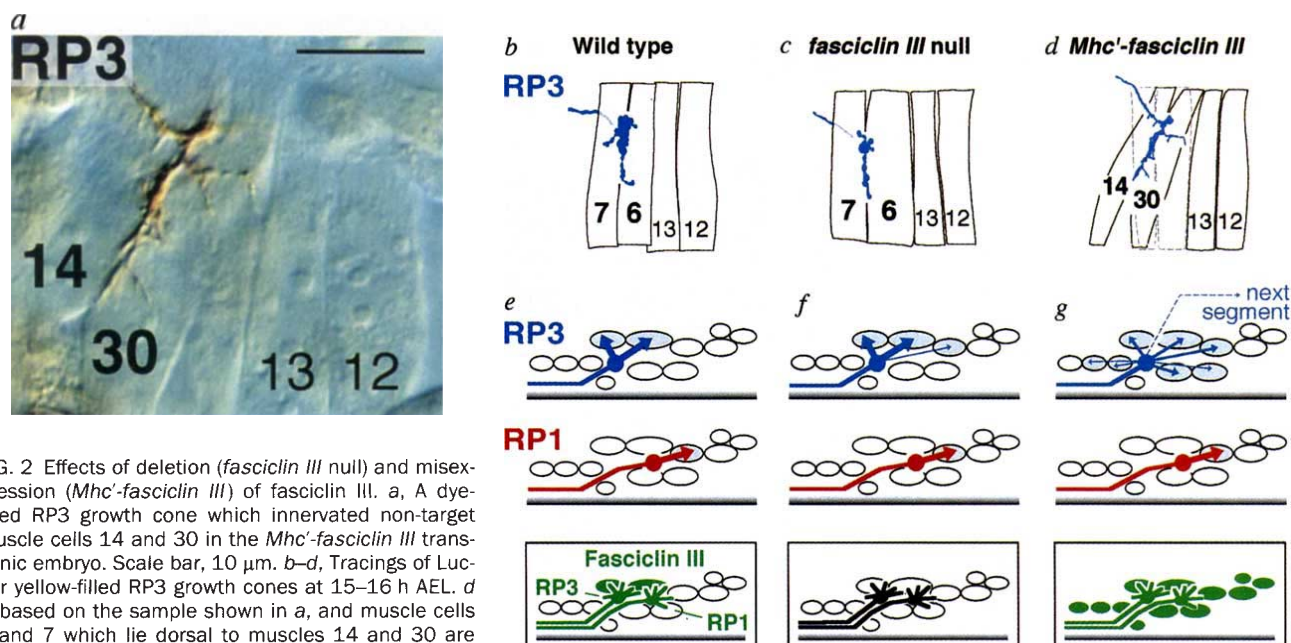


FIG. 2 Effects of deletion (*fasciclin III* null) and misexpression (*Mhc'-fasciclin III*) of fasciclin III. *a*, A dye-filled RP3 growth cone which innervated non-target muscle cells 14 and 30 in the *Mhc'-fasciclin III* transgenic embryo. Scale bar, 10 μ m. *b-d*, Tracings of Lucifer yellow-filled RP3 growth cones at 15–16 h AEL. *d* is based on the sample shown in *a*, and muscle cells 6 and 7 which lie dorsal to muscles 14 and 30 are indicated by broken outlines. *e-g*, Summary of target selections by individual RP growth cones. Likelihood of each muscle cell being innervated (from the homozygotes data in Table 1) is reflected in the relative thickness of the arrows extending from the growth cones. Shown inside box is the fasciclin III expression pattern in each experiment at roughly 12 h AEL. See Fig. 1a for muscle cell identities.

METHODS. Lucifer yellow was injected into a single RP motor neuron as described⁹. To ensure the identity of each dye-filled growth cone: (1) before dye injection we identified both RP1 and RP3 cell bodies

clearly within the CNS using a video system attached to the Nomarski optics-equipped microscope; (2) we inserted the dye-filled micropipette into the CNS about one cell diameter away from both RP cell bodies and penetrated either RP3 and RP1 without piercing through the other; and (3) following the dye injection confirmed that only a single RP cell body and its axon was being filled with the dye. The embryos were processed for anti-Lucifer yellow immunocytochemistry⁹.

one cell-bound target recognition molecule which can attract a specific growth cone to a single target cell, as envisioned by Sperry's 'chemoaffinity theory'⁵. □

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A new antigen receptor gene family that undergoes rearrangement and extensive somatic diversification in sharks

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IMMUNOGLOBULIN and T-cell receptor (TCR) molecules are central to the adaptive immune system. Sequence conservation, similarities in domain structure, and usage of similar recombination signal sequences and recombination machinery indicate that there was probably a time during evolution when an ancestral receptor diverged to the modern-day immunoglobulin and TCR^{1–3}. Other molecules that undergo rearrangement have not been described in vertebrates, nor have intermediates been identified that have features of both these gene families. We report here the isolation of a new member of the immunoglobulin superfamily from the nurse shark, *Ginglymostoma cirratum*, which contains one variable and five constant domains and is found as a dimer in serum.

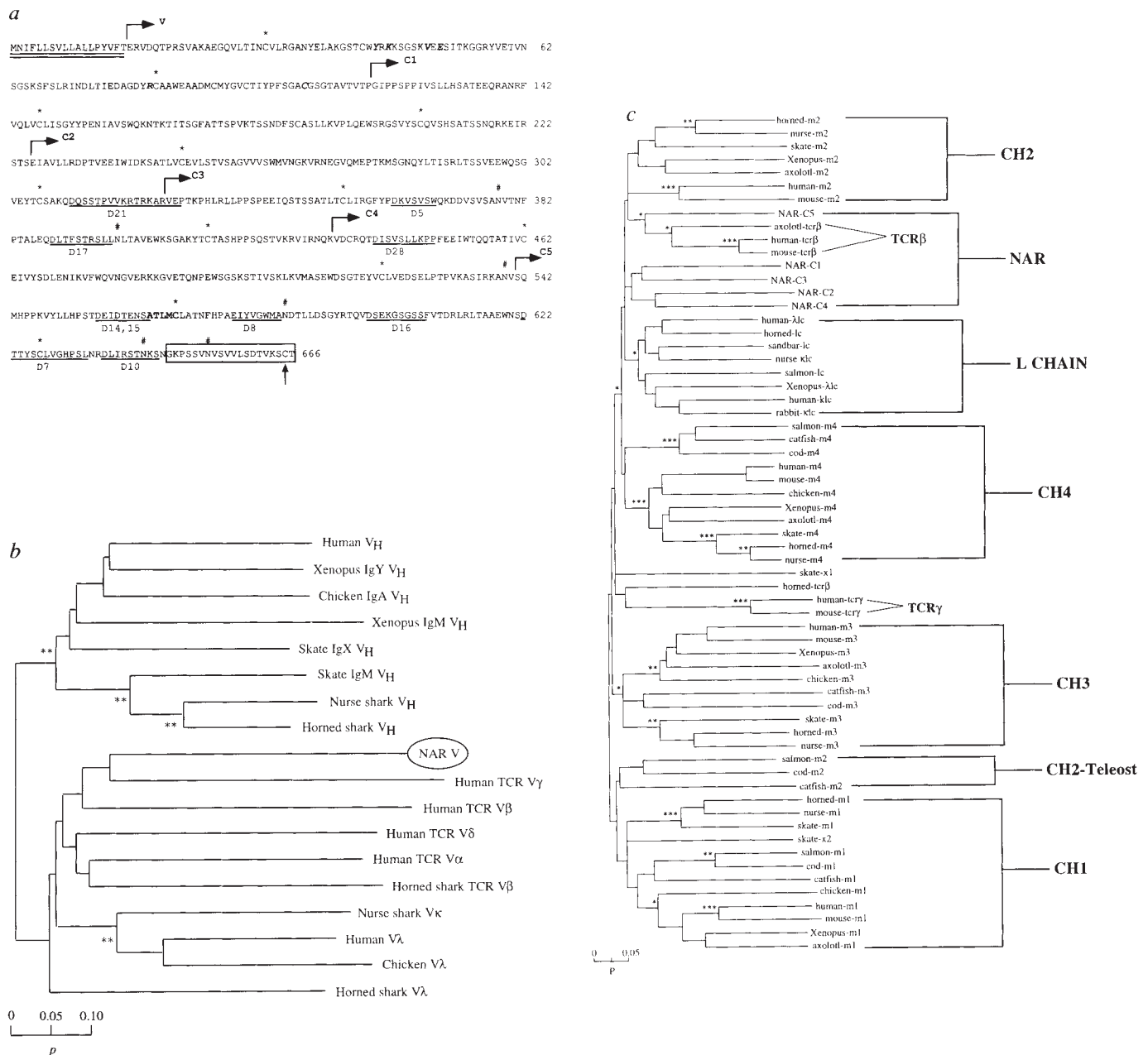


FIG. 1 Sequence of NAR clone 3-4 and phylogenetic trees using NAR domains. **a**, Sequence of clone 3-4. The putative signal sequence is double-underlined, and the positions of the domains are demarcated above the sequence. Cysteines that would take part in intradomain disulphide-bond formation are indicated by an asterisk, and potential glycosylation sites are indicated by #. The secretory tail is boxed, and the penultimate cysteine is indicated by an arrow. The sequence to which the primer CB2 binds is indicated in bold in C5⁴. Amino acids in the NAR V region which would be used for interdomain interactions are indicated in bold and italics. The location of peptides generated by Asp-N digestion of affinity-purified protein (see legend to Fig. 4) are underlined below the sequence. Amino-acid numbering is shown to the right of the sequence. **b**, Phylogenetic tree comparing the V region of NAR with immunoglobulin and TCR V regions. TCR, Ig L chain, and Ig H chain sequences are indicated, and the NAR V region is circled. Test of the hypothesis that branch length equals zero: *, $P < 0.05$; **, $P < 0.01$. **c**, Phylogenetic tree comparing the C domains of NAR with those of Ig and TCR. Each cluster of Ig CH and NAR regions is bracketed. Test of the hypothesis that branch length equals zero: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

METHODS. cDNA cloning, sequencing and phylogenetic tree construction. Preparation of the 1st strand cDNA template, oligonucleotide sequences, as well as the conditions used for PCR have been described

elsewhere⁴. A nurse shark spleen cDNA library was screened with the 700 bp PCR product as described previously⁴. Clone 3-4 was sequenced on both strands with Sequenase (US Biochemical) using standard protocols. Demarcation of the C domain boundaries was determined in part by analysis of genomic clones containing the C domains (Fig. 3; A.S.G. and M.F.F., unpublished data). TCR, Ig H and Ig L chain V and C regions from representative vertebrates were aligned with the CLUSTAL V program, and then were manually corrected. GenBank accession numbers for the V tree sequences are L16765, M92851, M23660, X03748, S12838, M29672, M20484, X15114, K00678, S40610, X06193, X06154, X04038, X15261, X57817 and S24679; accession numbers for the C tree sequences are X70168, X68700, M73062, M20584, X01613, X03690, M28601, M12815, X00232, M14157, L16765, M92851, M81314, M64307, X07784, M29674, M29672, M27230, X65260, S48652, X67301 and S15480 (from PIR database). Human κ and λ L chains and TCR γ C regions are from ref. 14. Sixty-four residues were used for the V region alignment after removal of the three CDRs and several residues found on loops, and sixty amino acids were used for the C region alignment. The neighbour-joining tree was based on the proportion of amino-acid difference (p)¹⁵. The significance of internal branches was tested by Rzhetsky and Nei's method¹⁶.

FIG. 2 Alignment of V and C1 region sequences obtained from cDNA clones. *a*, Alignment of the V domains. The deduced amino-acid sequences of the cDNA clones are aligned with the predicted amino-acid sequence of clone 3-4. Dashes (—) indicate identity with amino acids found in clone 3-4. The sequences are grouped into the two classes described in the text. The location of the putative β -strands is shown above the alignment, which is based in part on the known structure of Ig V domains. Strand 'G' corresponds to the J segment found in Ig and TCR V domains. The location of the predicted CDRs is also indicated above the sequences. No attempt was made to align the sequences in CDR3, and the underlined sequences in CDR3 represent potential D regions. Clones S1A, 5A and 2A are identical in sequence to clones 3-4, 4D and SH1D, respectively. *b*, Alignment of the C1 domains. Each of the C1 domain sequences fell into two classes, which correlated with the V domain type I and type II designations. We have not sequenced the entire C1 domain of clones 1I and 2A; however, the pattern of substitutions observed so far is identical to that seen in other type II clones. *c*, Alignment of V region sequences from cDNA clones. The predicted amino-acid sequences of cDNA clones derived from genomic cluster JIRB5 (Fig. 3d) are shown. Dashes indicate identity with amino acids found in the germ-line sequence. A slash (/) in 3L represents a gap introduced because of a loss of three nucleotides. A full stop in clone 3G1 indicates a stop codon because of a loss of one nucleotide. Clones 12C and 12P are identical in sequence. The location of the β -strands is indicated above the alignments.

METHODS. Nurse shark spleen cDNA libraries constructed in Uni-Zap XR (Stratagene) were screened under low-stringency conditions (30% formamide hybridization solution), and clones were sequenced as described above. Alignments were made by visual inspection of the sequences.

c

	Leader	A	B	CDR1	C	CDR2	D	E	F
Germine	MNIFLLSVLLALLPYVFTARVDQTPRSVTKETGESLTINCLVRDASYALGSTCWRKKS	SGSTNEESISKGRYVETVNSGSKSFLRLINDLTVEDGGTYRCGV							
5NC	-----	-----	-----	-----	-----	-----	-----	-----	-----
7A	-----	-----	-----	-----	-----	-----	-----	-----	-----
3NG	-----	-----	-----	T-TN	-----	L-----	-----	E-----	H-----
3M	-----	E-----	-----	-----	L-----	R-----	-----	C-----	-----
3L	-----	R-----	-----	RSV-----	-----	I-----	/A-----	T-----	I-----
3G1	-----	-----	S-----	L-----	S-L-----	R-E-----	-----	L-----	-----
3G	-----	TE-----	TD-----	R-----	Q-AD-----	F-----	-----	V-----	-----
2T	-----	F-----	-----	D-----	T-----	P-R-----	P-----	R-ER-----	A-----
2L	-----	-----	G-----	N-S-A-----	-----	-----	-----	-----	-----
2K	-----	FV-----	G-----	N-G-----	R-----	SP-----	K-----	A-D-----	A-----
2A	-----	V-----	L-----	A-----	S-----	L-----	R-----	-----	R-----
2E	-----	-----	V-LVK-----	G-----	-----	K-----	I-----	A-----	N-----
1I*	-----	A-----	-----	V-----	T-DVAN-----	F-----	I-----	RI-R-----	T-KA-----
12J	-----	-----	K-----	-----	FGP-----	R-----	P-----	L-D-----	V-----
3NB	-----	-----	YH-----	NYN-----	P-G-S-----	RLPR-----	F-----	A-D-----	T-----
3NK*	-----	-----	K-----	N-----	TLQ-----	DD-----	E-----	F-----	V-----
5NB	-----	-----	ER-----	R-----	NN-----	R-----	IR-----	I-----	K-----
1ND*	-----	V-----	-----	EL-----	V-----	-----	-----	-----	-----
9K	-----	C-----	A-----	-----	A-V-----	RFDG-----	E-----	F-----	II-----
3S	-----	-----	-----	-----	-----	P-----	-----	QE-----	K-----
9F	-----	-----	-----	N-----	-----	-----	T-----	Q-----	-----
3N	-----	-----	-----	N-P-----	F-----	L-----	-----	S-----	F-----
12A	-----	G-----	-----	A-----	H-R-----	G-R-----	G-PVPDR-----	YM-L-----	L-----
10N	-----	-----	S-----	-----	D-----	-----	KYVG-----	VM-----	-----
2P	-----	-----	L-----	-----	TD-EIRP-----	T-----	D-----	VR-----	D-----
3H1	-----	-----	V-L-----	T-----	-----	A-----	N-----	R-----	R-----
2H	-----	-----	T-----	R-----	-----	N-----	G-----	I-----	GS-----
12C/12P	-----	-----	T-----	L-----	I-----	TN-----	VR-----	L-----	R-----
7B	-----	-----	A-----	-----	D-----	E-----	T-----	SD-KR-----	K-----
12K	-----	-----	-----	-----	-----	-----	-----	L-----	-----

a

	Leader	A	B	CDR1	C	CDR2
S1A, 3-4	MNIFLLSVLLALLPYVFTARVDQTPRSVAKARQQVLTINCLVRGANYELAKGSTCWRKKS	SGSKVEES				
1C	-----	A-----	T-ET-ES-----	D-S-----	L-H-----	TK-N-----
5H	-----	V-----	T-ET-ES-----	D-I-----	TV-----	TAV-----
3-9	-----	-----	T-ET-ES-----	D-TS-----	L-N-----	S-----L-TR-I-----
3-10	-----	A-----	T-ET-ES-----	D-T-----	SL-----	-----TN-----
5F*	-----	A-----	T-ET-ES-----	A-----PSL-----	LE-----	L-----TN-L-----
5D	-----	W-L-----	SA-T-ET-ES-----	DSFQ-----	SLAG-----	FTR-----RS-R-----
2D	-----	N-----	T-ET-ES-----	E-P-----	L-K-----	-----TN-Q-----
4J	-----	A-----	QTIT-ET-ES-----	D-----	L-R-----	-----TH-V-----
1A	-----	-----	H-IT-ET-ES-----	DSAC-----	GLSNAQ-----	ER-----TK-N-----
1-3	-----	A-----	QEIT-ET-ES-S-----	DS-C-----	LP-Y-N-----	-----TN-T-----
1I	-----	V-----	QTIT-ET-ES-----	DS-C-----	LS-Y-----	-----TN-K-----
1N	-----	A-----	QTIT-ET-ES-----	NSAC-----	LST-Y-----	A-----TN-----
4-1	-----	V-----	QTIT-ET-ES-----	DS-C-----	PLSD-S-----	-----TN-S-----
4F*	-----	A-L-----	QTIT-ET-ES-----	DSDC-----	SFG-S-----	-----SI-K-----
5A, 4D	-----	N-L-----	H-IT-ET-ES-----	DS-C-----	SLI-R-----	-----TN-S-----
2A, SH1D	-----	-----	QTIT-ET-ES-----	P-DM-C-----	LS-Y-----	-----SN-----

b

	CDR2	D	E	F	G					
S1A, 3-4	ITKGGRYVETVNSGSKSFLRLINDLTIEDAGDYRCAAEAAADCMYGVCTIYYPF				SGACGSGTAVTVTF					
1C	-----	VQ-----	V-----	G-T-----	GGSLYNMCSCETDVLPI	A-----A-----N-----				
5H	-----	S-----	V-----	G-T-----	GAISYQCDPVCAAN	YV-----D-----N-----				
3-9	-----	A-----	V-----	G-T-----	GVGQYRGCPYFLCSHLS	YAG-----D-----V-----N-----				
3-10	-----	S-----	V-----	G-T-----	GVGNACGGDKGCGDYRLCSYIDE	-----D-----N-----				
5F*	-----	ST-----	K-----	V-----	G-T-----	TVGVWAGAYCGEVCKLT	-----D-----F-----N-----			
5D	-----	RA-----	-----	K-----	V-----	G-T-----	GVAGTHSGCALCSFIQIE	AT-----E-----N-----		
2D	-----	S-----	EL-----	V-----	G-T-----	GAMPSSWGHGCSWDWAVGAYAY	-----V-----D-----N-----			
4J	-----	E-----	-----	M-----	V-----	S-TF-----	GVWQWGTCDRRCVHNSR	D-----G-----V-----N-----		
1A	-----	SQ-A-----	RE-----	V-----	S-S-----	MASTWYTDIEGGLGTPCNRQH	DVY-AD-V-----N-----			
1-3	-----	S-----	V-----	S-T-----	KVYRKNWYDCGLELDWIY	-----	VY-G-----G-----N-----			
1I	-----	VS-----	V-----	S-T-----	KTGMLHDCDWSYD	-----	VY-G-----V-----N-----			
1N	-----	S-----	V-----	S-T-----	MVLRCAWYLD	-----	VY-G-----V-----N-----			
4-1	-----	MS-----	V-----	S-T-----	KLAWYGTGCVINGRFLYD	-----	VY-G-----V-----N-----			
4F*	-----	G-----	Q-----	D-----	NA-----	V-----	S-T-----	KVPWDGDNVD	-----	VY-----VL-----N-----
5A, 4D	-----	S-----	-----	R-----	V-----	S-T-----	GGRSGYGCIFYE	-----	VY-G-----V-----N-----	
2A, SH1D	-----	S-----	-----	V-----	S-T-----	RLWCMTGRSPAYN	-----	Y-G-----V-----N-----		

Analyses of complementary DNA clones show extensive sequence diversity within variable domains, which is generated by both rearrangement and somatic diversification mechanisms. Our results suggest that rearranging loci distinct from immunoglobulin and TCR have arisen during evolution.

Using the immunoglobulin-superfamily-specific primer CB2 for 3' rapid amplification of cloned ends (RACE), a 700-base-pair (bp) band was amplified from nurse shark spleen cDNA that had amino-acid similarities to the immunoglobulin superfamily C1-SET domains^{4,6}. The polymerase chain reaction (PCR) product was used as a probe to screen a spleen cDNA library, and a 2.4-kilobase (kb) cDNA clone (3-4) was isolated. The deduced amino-acid sequence of clone 3-4 is shown in Fig. 1a. We have called this molecule new or nurse shark antigen receptor (NAR). A potential leader sequence is followed by a domain most similar to immunoglobulin superfamily V-SET domains⁶. Although amino acids involved in forming the core of immunoglobulin and TCR V regions are conserved in NAR,

residues used for interactions between immunoglobulin V domains are not well conserved (bold and italicized residues in Fig. 1a), suggesting that NAR V domains do not pack in a similar fashion to those in immunoglobulin and TCR⁷. The remaining sequence consists of five immunoglobulin superfamily C1-SET domains. The carboxy terminus of clone 3-4 is similar (50–60% in amino-acid sequence) to the tail region of secretory IgM and IgA and contains the penultimate cysteine that in IgM and IgA binds J chain (boxed in Fig. 1a). Northern blotting detected highest NAR expression in the spleen, with a faint signal in liver samples; transcripts were not detected in brain, kidney, heart, testis or gill samples (data not shown).

Phylogenetic trees were constructed to evaluate the relationship of NAR domains with immunoglobulin and TCR V and C domains. The NAR V region clusters with TCR V regions (Fig. 1b). NAR C domains 1–4 cocluster rather than group with potentially homologous IgM CH domains (Fig. 1c), and C5 clusters with TCR C β domains (Fig. 1c). NAR does not cluster

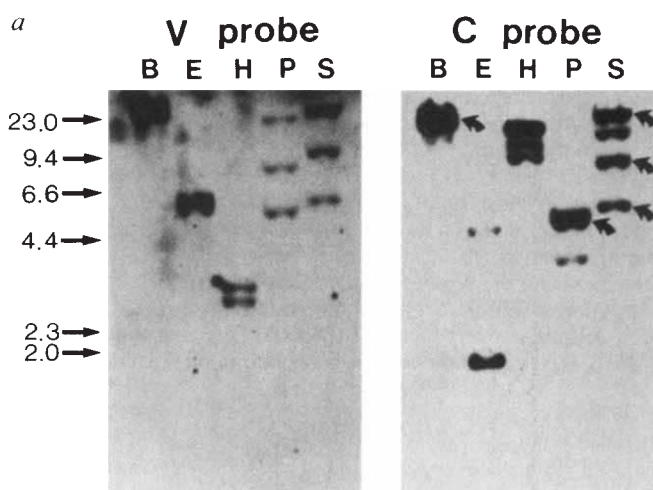
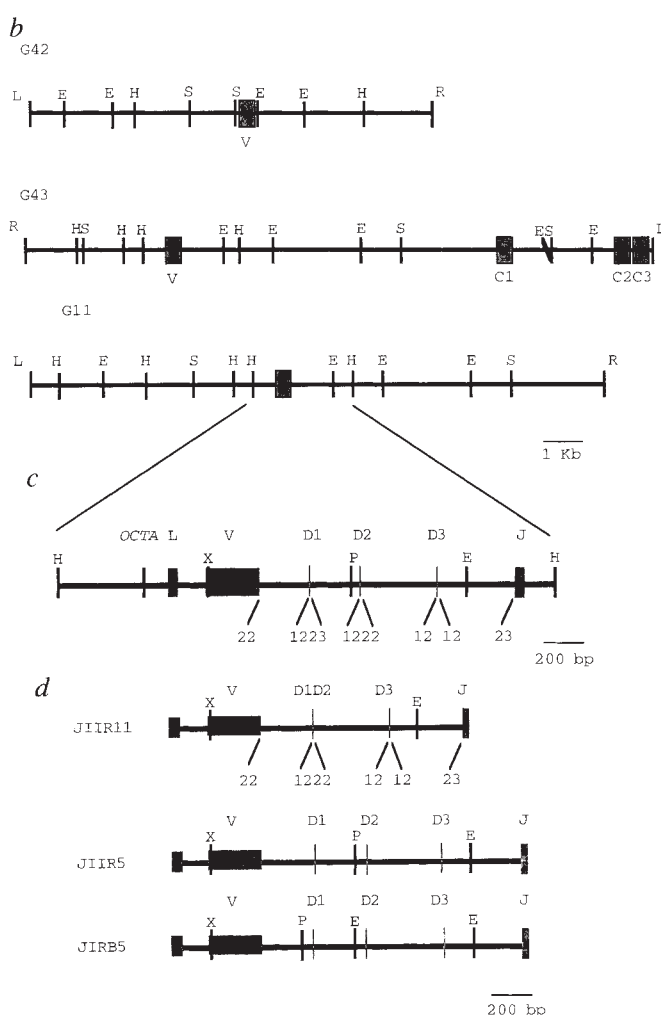


FIG. 3 Genomic analyses of NAR. **a**, Southern blotting using V- and C1-specific probes. The V probe was hybridized under reduced stringency conditions. V and C1 probes were sequentially hybridized to the same filter. Arrows indicate bands that hybridize to both probes, suggesting a possible linkage within the genome. Size standards (kb) generated by digestion of λ DNA with *Hind*III are indicated at the left of the figure. B, *Bam*HI; E, *Eco*RI; H, *Hind*III; P, *Pst*I; S, *Sac*I. **b**, Restriction map of genomic clones G11, G43 and G42. The location of the V and C regions are indicated within these clones. R and L indicate the positions of the right and left arms of the phage, respectively. **c**, Restriction map of the 2.5 kb *Hind*III V-hybridizing fragment. The locations of the leader peptide, V, D and J regions are indicated above the map, and the spacer units between the heptamer-nonamer is indicated below the map. X, *Xho*I. **d**, Genomic clusters amplified by PCR. The location of V, D and J segments are shown for each clone. A D region in JIIR11 (D1–D2) may represent a germ-line join of D1 and D2 segments.

METHODS. Erythrocyte genomic DNA (10 μ g) was digested with the appropriate restriction enzymes and electrophoresed on a 0.8% agarose gel. Southern blotting was as described previously^{4,17}. Probes specific for (amino acids 4–85 in Fig. 1a) and C1 (amino acids 118–227) were generated by PCR using clone 3–4 as a template and were labelled to a specific activity of $2\text{--}3 \times 10^9$ c.p.m. μ g⁻¹ DNA with a random-primed labelling kit (Boehringer-Mannheim). Hybridization and washing under stringent conditions with the C1 probe was as described⁴, and the V probe was hybridized in similar solution to the C1 probe except that it contained 30% instead of 50% formamide. The V-specific hybridization filter was washed with $1 \times$ SSC, 0.1% SDS for 60 °C twice for 20 min each before autoradiography with Kodak X-Omat film. Exposure times for the blots were 48 h. A shark genome phage library (4×10^6 PFU) was screened with a V probe under reduced stringency conditions. Mapping was done using a combination of single-double restriction enzyme digests as well as partial digests (Lambda-Map, Promega). The order of restriction enzyme sites was confirmed in some cases by cloning fragments into pBS SK+ (Stratagene). The 2.5 kb



*Hind*III hybridizing V fragment of G11 was cloned into *Hind*III-digested pBS and was further subcloned by restriction with various enzymes and ligation to appropriately cut pBS. Sequencing was done on the entire V-J region of G11 in order to determine the location of the rearranging gene segments. Thirty cycles of PCR were done on 1 μ g of erythrocyte genomic DNA using a combination of NS2L (5'-ATGAATATTTCTGCTKTCAGT-3') and either JIR (5'-ACAGTCACGRCAGTCCRTCCRC-3') or JIIR (5'-ACAGTCACGACAGTGCCACCTCCRTA-3') primers. Cycling conditions were 94 °C for 1 min, 48 °C for 2 min, and 72 °C for 3 min. Cloning and sequencing of PCR products was done as described elsewhere⁴. Southern blotting has confirmed the existence of clusters JIIR5 and JIRB5; the authenticity of JIIR11 remains to be established.

with the recently reported shark TCR sequence (Fig. 1*b, c*). Together, the V and C trees suggest that NAR arose from a two-domain TCR/L chain-like ancestor and evolved its own set of C domains. Isolation of NAR from other species will be needed to test this hypothesis.

Characterization of additional cDNA clones showed that the predicted amino-acid sequences of the V domains differed from clone 3-4 (Fig. 2*a*). The V sequences are classified into two groups (type I and II) based on three criteria: (1) a cysteine found in strand C in type I V regions; (2) the motif QTIT in strand A of type II V regions; and (3) a cysteine in the potential J region of type I, but not type II V regions (Fig. 2*a*). CDR3 is heterogeneous in both length and sequence among cDNA clones and contains 1-4 cysteines (Fig. 2*a*). Short sequence identities in CDR3 found between clones may correspond to diversity (D) regions (underlined in Fig. 2*a*). The sequences of the C1 domains also fall into two classes, correlating with the type I and II designation assigned for V domains (Fig. 2*b*). These data suggest that NAR undergoes rearrangement.

Southern blotting using NAR V- and C1-specific probes detected only a few bands (Fig. 3*a*), suggesting that there are only a few NAR genes. Several V-hybridizing genomic clones have been isolated (Fig. 3*b*). G11 and G43 may represent alleles as they have similar restriction maps yet contain a *HindIII* polymorphism upstream of the V region (Fig. 3*b*). Clone G42 contains a different V region to that in G11/G43. Two other genomic clones contained the same V region as G11/G43; the low number of clones isolated (5) is consistent with the Southern blotting data. C1-3 domain probes hybridize to clone G43, confirming the V-C linkage suggested by Southern blotting.

The G11 V region contains an octamer upstream of the presumed translational start site, a regulatory element found in the

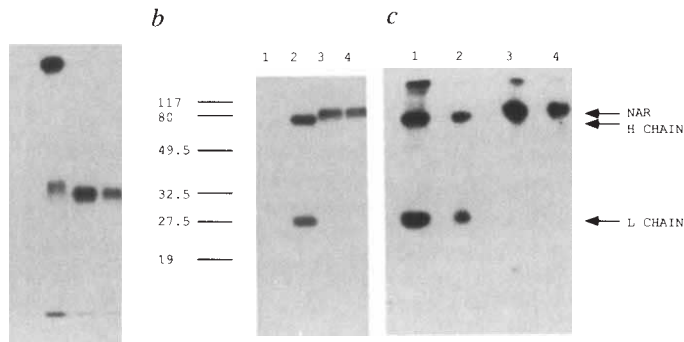
promoters of immunoglobulin genes⁸. A short intron of 134 bp is present within the leader exon, similar to that found for immunoglobulin and TCR loci. A recombination signal sequence (RSS) containing 22 nucleotides is found downstream of the V region. Further subcloning and sequencing of the G11 2.5 kb *HindIII* fragment revealed three D and one J segments downstream of the V region (Fig. 3*c*). The three D regions are flanked by RSS containing either 12 or 22/23 nucleotides; it is possible to use either one, two or all three D regions for deletion V(D)J rearrangements.

Further study of somatic diversification was addressed by amplifying V-D-J clusters from genomic DNA by PCR and constructing cDNA libraries from the individual used for Southern blotting (Fig. 3*a*). Genomic clones JIR5 and JIR11 contain type II V regions, and JIRB5 has a type I V region (Fig. 3*d*). Sequence analyses of V regions from 45 cDNA clones revealed that all were type I. The amino-acid sequences of cDNA clones derived from cluster JIRB5 or a closely related allele are shown in Fig. 2*c*. Two cDNA clones (5NC and 7A) encode V regions nearly identical to the germ-line sequence; all other clones differ in sequence from JIRB5 (Fig. 2*c*). Every cDNA clone isolated has a different V region sequence yet only a few V-hybridizing bands are detected by Southern blotting, strongly suggesting that NAR undergoes extensive somatic diversification, probably through mutation.

We have generated monoclonal antibodies specific for a partially purified fraction of 7S IgM. One of these antibodies (CB8) immunoprecipitates a molecule distinct from 7S IgM; subsequent protein purification and peptide sequencing showed that the peptides were present in the predicted amino-acid sequence of NAR (see Fig. 1*a* for the location of peptides). Second generation antibodies were produced to characterize NAR further. Electrophoresis under non-reducing conditions (Fig. 4*a*) showed

FIG. 4 SDS-PAGE analysis of immunoprecipitations using various mAbs. *a*, Immunoprecipitations under non-reducing conditions. Radiolabelled nurse shark serum was immunoprecipitated with either mAb TB17 (α Xenopus class I, lane 1), a combination of mAbs CB11 and CB16 (α shark IgM, lane 2), mAb GA12 (α NAR, lane 3) or mAb GA13 (α NAR, lane 4). Immunoprecipitates were resolved on a 5% polyacrylamide gel. *b*, Immunoprecipitations under reducing conditions. Nurse shark serum was immunoprecipitated with mAb TB17 (lane 1), mAbs CB11 and CB16 (lane 2), mAb GA12 (lane 3) or mAb GA13 (lane 4), and β -mercaptoethanol was added to the samples before resolution on a 11% polyacrylamide gel. The apparent migration of prestained *M_r* standards (BioRad) are shown to the left of each figure. *c*, Excision of bands from *a*, reduced, and resolved on an 11% polyacrylamide gel. Bands corresponding to 19S and 7S IgM (*a*, lane 2), GA12 (*a*, lane 3), or GA13 (*a*, lane 4) were excised from the gel and resolved on a second gel after reduction. The order of the lanes are 19S IgM (lane 1), 7S IgM (lane 2), GA12 (lane 3) and GA13 (lane 4). Arrows indicate the positions of H chain, L chain and NAR.

METHODS. Mice were immunized with a partially purified fraction of 7S IgM or affinity-purified NAR, and fusions were as described using X63-Ag8.653 as the fusion partner¹⁸. Production of mAb TB17 has been described elsewhere¹⁹. Nurse shark serum was labelled with ¹²⁵I using the chloramine T technique²⁰. Immunoprecipitations were done by the solid-phase immunoprecipitation technique²¹. The 96-well plates were pre-incubated overnight with 2.5 μ g of goat anti-mouse Ig (Fisher-Biotech), after which 100 μ l of mAb supernatant was added for at least 5-6 h at room temperature. Radiolabelled sera (1-2 μ l, corresponding to $\sim 10^6$ c.p.m.) were added to the wells and were incubated at 4 °C overnight. The wells were washed 5 times with NET-N solution (150 mM NaCl, 5 mM EDTA, 50 mM Tris pH 8.0, 0.5% Nonidet P-40, and 1 mg ml⁻¹ ovalbumin) and then 4 times with NET-N solution (NET-N without ovalbumin). Laemmli sample buffer (30 μ l) was then added to the wells²², and samples to be reduced had β -mercaptoethanol added. The samples were boiled for 5 min, after which they were loaded onto the gels. Bands excised from the 5% polyacrylamide gel were loaded onto an 11% polyacrylamide gel, and a reducing solution (1% low melting point agarose, 11% glycerol, 2.5% SDS, 150 mM Tris, pH 7.0, 0.1%



bromophenol blue, 3% β -mercaptoethanol) was overlaid. After an incubation of 15 min, electrophoresis was done. Autoradiography was done with Kodak X-Omat film for 48 h, except for *c*, where exposure time was 8 days. mAb CB8 was coupled to protein G following the method of Schneider²³. NAR was purified from nurse shark serum by incubating the beads overnight with serum at 4 °C with rotation. Gel filtration was then done with a Superose 12 HR 10/60 column (Pharmacia) to remove the contaminating 19S IgM. The sample was treated with β -mercaptoethanol, alkylated with 4-vinylpyridine, and desalted using an Aquapore RP-300 column (2.1 $\times$ 20 mm, Brownlee). The sample was then lyophilized and resuspended in 0.05 M NH₄HCO₃, pH 8.0, and 0.5 μ g of endoproteinase Asp-N (Boehringer-Mannheim) was added. Digestion was done for 10 h at 30 °C, after which the sample was lyophilized and resuspended in 0.1% TFA. The peptides were fractionated by HPLC on an Aquapore butyl column (2.1 $\times$ 30 mm, Brownlee) and were microsequenced using an Applied Biosystems 475A Protein Sequencer. The sequences of the peptides are shown underlined in Fig. 1*a*; the sequence obtained for D5 peptide (DKVSVSCET) does not match the predicted amino-acid sequence in clone 3-4 exactly and may represent errors in protein sequencing or expression of another allele/locus. The Asn in peptide D10 was detected without any appreciable loss in yield, suggesting that no N-linked carbohydrate is found at this position.

that IgM (lane 2) has a slower electrophoretic mobility than NAR (lanes 3 and 4). The reduced NAR appears as a single band of 80–85K (Fig. 4b, lanes 3 and 4). No bands in the L-chain region are detected for NAR, even after reduction of the non-reducing immunoprecipitations (Fig. 4b, c, lanes 3 and 4); furthermore, no L chains were detected with purified NAR protein stained by Coomassie brilliant blue or by metabolic labelling (data not shown). Higher multimeric forms, or an association with J chain are not observed in immunoprecipitations (Fig. 4a, lanes 3 and 4, and A.S.G. and M.F.F., unpublished observations).

Sharks are the most primitive vertebrates in which an adaptive immune system has been described. There is a lack of affinity maturation for shark IgM during immune responses, graft rejection is of a chronic nature, and no true effector functions of T

cells have been described^{9,12}. Because NAR is a secreted molecule and undergoes somatic mutation, it is most probably selected to bind to antigen in solution like immunoglobulin. It is unknown whether the high frequency of mutations (6% for the sequences in Fig. 2c) are introduced as a consequence of antigenic stimulation or rather to generate the NAR repertoire similar to the process described for B cells in sheep ileal Peyer's patches¹³. The finding of another molecule undergoing rearrangement and extensive diversification suggests that sharks use other antigen receptors besides immunoglobulin and TCR during immune responses. It remains to be determined whether NAR homologues will be found also in other vertebrates. Our data suggest that additional antigen receptors may be present in some vertebrates and play specialized roles in their immune systems. □

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ATP-dependent inositide phosphorylation required for Ca^{2+} -activated secretion

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REGULATED fusion of secretory granules with the plasma membrane in secretory cells requires ATP, Ca^{2+} and cytosolic Ca^{2+} as well as membrane proteins. ATP-dependent steps in Ca^{2+} -activated secretion from PC12 cells require three cytosolic PEP proteins (priming in exocytosis proteins, PEP1–3)^{5,6}, the identity of which will provide insights into the required ATP-using reactions. PEP3 was recently identified as phosphatidylinositol transfer protein (PtdInsTP)⁶, and here we report that PEP1 consists of the type I phosphatidylinositol-4-phosphate 5-kinase (PtdInsP5K). The roles of PEP3/PtdInsTP and PEP1/PtdInsP5K in sequential phosphoinositide recruitment and phosphorylation explains their synergistic activity in ATP-dependent

priming. Moreover, inhibition of Ca^{2+} -activated secretion by PtdIns(4,5) P_2 -specific antibodies and phospholipase C implies that 5-phosphorylated inositides play a novel, necessary role in the regulated secretory pathway. The results indicate that lipid kinase-mediated phosphorylation is an important basis for ATP use in the exocytotic pathway.

ATP-dependent, Ca^{2+} -activated secretion proceeds through sequential ATP-dependent priming and Ca^{2+} -activated triggering steps for which distinct cytosolic proteins are required⁵. Three rat brain cytosolic PEP proteins with apparent M_r s of 500K (PEP1), 120K (PEP2) and 20K (PEP3) stimulate the ATP-dependent priming of Ca^{2+} -activated noradrenaline secretion from semi-intact PC12 cells (Fig. 1b). Previous studies⁶ identified PEP3 as PtdInsTP but did not specify the role of this protein in priming, which could involve phosphatidylinositol (PtdIns) and/or phosphatidylcholine (PtdC) transfer⁷ or the regulation of PtdC metabolism as suggested for the catalytically similar yeast SEC14p^{8,9}. Phospholipid transfer by PtdInsTP does not require ATP yet PtdInsTP was required for an ATP-dependent step in regulated secretion⁵. Hence, it was possible that PtdInsTP transfers PtdIns to, or activates PtdIns in, a membrane for phosphorylation by ATP-dependent phosphoinositide kinases, because PtdInsTP catalyses a rate-limiting step in ATP-dependent PtdIns(4,5) P_2 synthesis¹⁰. Priming by PEP3/PtdInsTP and PEP1 (Fig. 1b) in semi-intact PC12 cells was accompanied by increased PtdIns(4,5) P_2 formation (Fig. 1c). This correspondence suggested that the synthesis of PtdIns(4,5) P_2 or a related lipid may be the biochemical reaction underlying the priming of exocytosis.

Mammalian phosphoinositide 4-kinases are predominantly membrane-associated proteins¹¹, whereas PtdInsP5Ks are soluble and extrinsic membrane proteins¹². PtdInsP5K activity in gel-filtered rat brain cytosol eluted in a single peak coincident with PEP1 (Fig. 1d), indicating that PEP1 may be PtdInsP5K.

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The type I isoform of PtdInsP5K detected by western blotting (not shown) eluted at the same position. PEP3/PtdInsTP fractions were devoid of PtdInsP5K activity (Fig. 1d) and immunoreactivity (not shown) but nonetheless stimulated PtdIns(4,5)P₂ formation (Fig. 1c). It is likely that PEP3/PtdInsTP stimulates

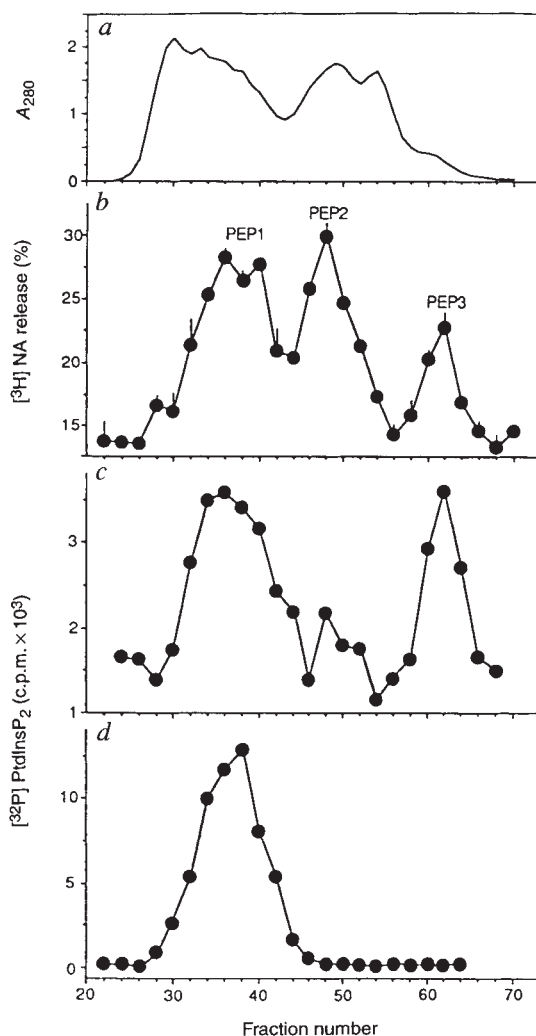


FIG. 1 Resolution of rat brain cytosolic priming factors by gel filtration chromatography. *a*, Protein; *b*, priming activity; *c*, PtdIns(4,5)P₂ formation in semi-intact cells; *d*, PtdInsP5K activity.

METHODS. Rat brain cytosol was prepared and gel filtered on Superose 12 (Pharmacia) in K-Glu buffer (20 mM HEPES, 120 mM potassium glutamate, 20 mM potassium acetate, 2 mM EGTA, pH 7.2) as previously described^{5,6}. Fractions were assayed (in duplicate) for priming activity in cytosol-depleted permeable PC12 cells as previously described^{5,6}. Briefly, semi-intact cells were incubated with column fractions and 2 mM Mg-ATP for 30 min at 30 °C, chilled and washed by centrifugation. Three-min triggering reactions at 30 °C were conducted with rat brain cytosol and sufficient CaCl₂ to establish 1 μM free Ca²⁺. Under these conditions, the extent of Ca²⁺-dependent [³H]noradrenaline ([³H]NA) secretion during triggering reflects priming activity⁵. In the assay of *b*, maximal priming with cytosol resulted in 48% [³H]NA release. PtdIns(4,5)P₂ formation in semi-intact cells was determined under priming conditions except that incubations were supplemented with 5 μCi of [³²P]ATP (final specific activity 12.5 mCi mmol⁻¹). Reactions were stopped by chilling and centrifugation, and cell pellets were dissolved in 1% Triton X-100. Phospholipids were extracted and analysed for PtdIns(4,5)P₂ content on tartrate-treated TLC plates using chloroform:methanol:H₂O:15 M NH₄OH (90:90:20:7) as the mobile phase and autoradiography to detect ³²P-labelled lipids. PtdInsP kinase assays were conducted as described¹² using PtdIns(4)P (Sigma) micelles as substrate and the above TLC system for analysis and quantification of ³²P-labelled PtdIns(4,5)P₂.

PtdIns(4,5)P₂ synthesis in the absence of added PtdInsP5K by recruiting PtdIns for phosphorylation by resident phosphoinositide kinases present on the semi-intact cell membranes¹⁰, because semi-intact cells retain 45% of the cellular type I PtdInsP5K (not shown). Of the cellular PEP3/PtdInsTP, 15 per cent was also retained by semi-intact cells, which probably accounts for the ability of PEP1 to prime submaximally in the absence of added PEP3/PtdInsTP (Fig. 1b). Our finding that PEP1 and PEP3/PtdInsTP exhibit mutually synergistic priming activities⁶ could be accounted for by the identity of PEP1 with PtdInsP5K because PEP1 and PEP3 would be components of a sequential pathway of polyphosphoinositide synthesis.

PEP1 was subjected to further purification and found to co-purify with the type I PtdInsP5K at each stage. On phosphocellulose, priming activity eluted sharply at 0.6 M NaCl with a shoulder at 0.65 M, indicative of two PEP1 isoforms (Fig. 2b), which precisely co-eluted with two isoforms of PtdInsP5K (Fig. 2c) that correspond to 90K (type Ib) and 68K (type Ia) proteins (Fig. 2d). The specific activity of the 90K isoform was substantially greater for both priming and for PtdIns(4)P phosphorylation. Mono Q anion exchange chromatography (Fig. 2e-g) also revealed identical co-elution of PEP1 priming activity with two PtdInsP5K isoforms (detected as PtdIns(4,5)P₂ formation in semi-intact cells), with the 90K type Ib isoform exhibiting greater priming and PtdInsP5K activities (not shown).

To establish definitively that PEP1 and PtdInsP5K activities reside in the same protein complex, affinity-purified antibody specific for the type I PtdInsP5K¹² was used in immunoprecipitation studies. The antibody progressively and completely depleted both priming (Fig. 3a) and PtdInsP5K (Fig. 3b) activities in parallel from a purified PEP1 fraction, whereas pre-immune IgGs were inert. The 90K type Ib PtdInsP5K protein in this preparation was depleted in parallel (Fig. 3c).

Because the subunit composition of the ~500K cytosolic PEP1/PtdInsP5K has not been elucidated^{5,12}, we probed the active site of the kinase with *p*-fluorosulphonylbenzoyl-adenosine (FSBA), an adenosine analogue that covalently modifies and irreversibly inactivates the ATP-binding site in many kinases¹³. The priming (Fig. 3d) and PtdInsP5K (Fig. 3e) activities of PEP1 were comparably inactivated by FSBA as anticipated if both activities reside with the same catalytic subunit of the complex.

A highly purified type I PtdInsP5K prepared from bovine erythrocytes¹² also catalysed ATP-dependent priming that was synergized by PEP3/PtdInsTP (Fig. 3f). In contrast, a purified erythroid type II PtdInsP5K¹⁴ entirely lacked priming activity (Fig. 3g). These results provide the first indication, to our knowledge, for an isoform-specific role for a phosphoinositide kinase in mammalian cells, that of the type I PtdInsP5K in membrane trafficking. Previously a yeast phosphoinositide kinase (PtdIns3-kinase, VPS34p) was shown to catalyse a step in vacuolar protein sorting¹⁵.

Our data demonstrate that the high *M_r* PEP1 required for priming contains the type I isoform of PtdInsP5K. It remains to be established whether the ~500K PEP1 is a homo-oligomer of catalytic PtdInsP5K subunits or contains additional subunits. ATP-dependent priming requires the FSBA-sensitive PtdInsP5K activity of PEP1, which implies that the formation of a 5-phosphorylated phosphoinositide is required for Ca²⁺-activated exocytosis. This conclusion was supported by studies with PtdIns(4,5)P₂-specific monoclonal antibodies¹⁶ and corresponding F_{ab} fragments, which block Ca²⁺-triggered secretion in semi-intact PC12 cells (Fig. 4a). A recombinant polyphosphoinositide-specific phospholipase C also inhibited Ca²⁺-activated secretion (Fig. 4b), further indicating a required role for polyphosphoinositides in regulated secretion.

The conclusion that phosphoinositide 5-phosphorylation is a critical reaction in ATP-dependent priming is compatible with previous studies that correlated polyphosphoinositide levels with the Ca²⁺ responsiveness of exocytosis in chromaffin cells¹⁷.

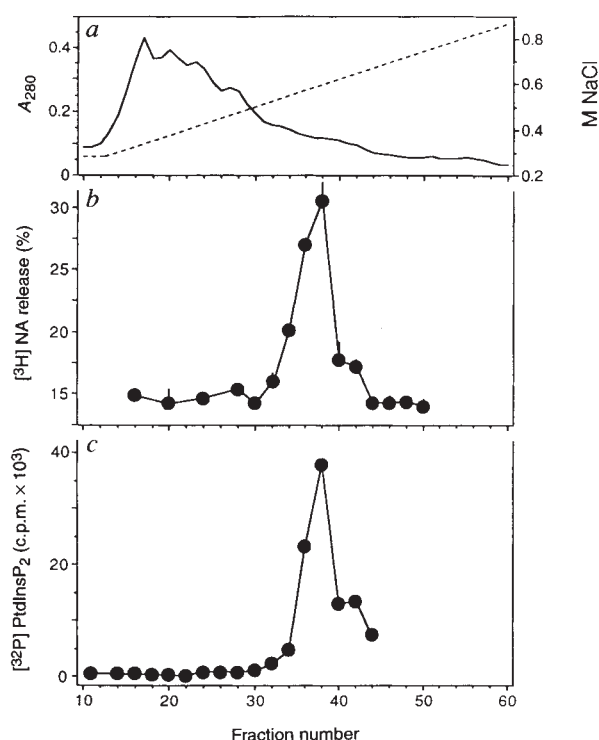
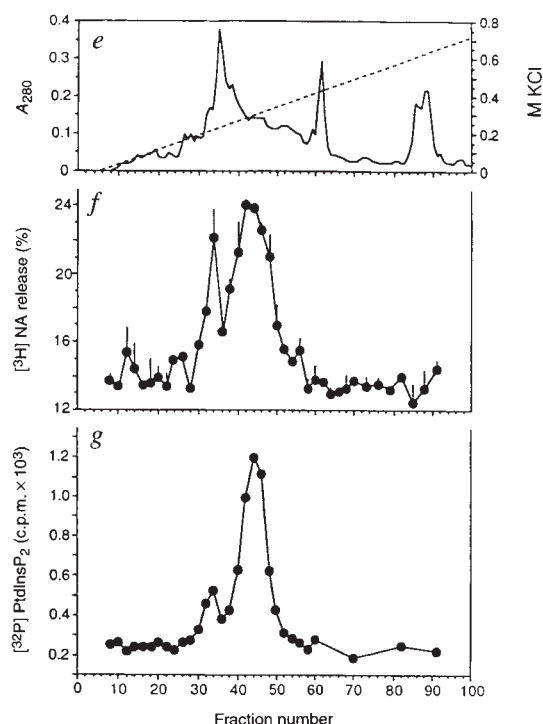
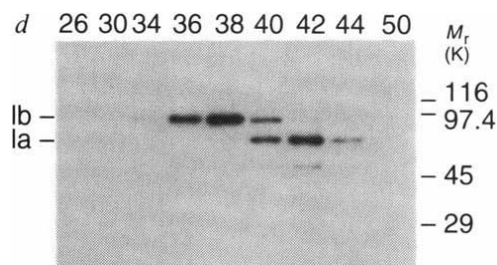


FIG. 2 Copurification of PEP1 and PtdInsP5K activities. Phosphocellulose column: a, protein and NaCl gradient; b, priming activity; c, PtdInsP5K activity; d, immunoblot of phosphocellulose fractions with anti-type I PtdInsP5K antibody; fraction numbers shown at top. Arrows denote 68K type Ia and 90K type Ib PtdInsP5K isoforms. Mono Q column: e, protein and KCl gradient; f, priming activity; g, PtdIns(4,5)P₂ formation in semi-intact cells.

METHODS. Cytosol from 50 rat brains prepared as in Fig. 1 was dialysed into buffer A (5 mM Na₂HPO₄, pH 7.5, 1 mM EDTA, 0.25 M NaCl, 10% glycerol, 2 mM β-mercaptoethanol, 0.1 mM PMSF) and passed through a 2.6 × 19 cm phosphocellulose column (Whatman P11). Flowthrough was gel filtered and tested for priming and PtdInsP5K activities as in Fig. 1. PEP2 and PEP3 were present in the flowthrough whereas PEP1 and PtdInsP5K were absent, indicating retention by phosphocellulose (not shown). The phosphocellulose column was eluted with an NaCl gradient from 0.25 to 1.0 M in buffer A. Mono Q chromatography: cytosol from 5 rat brains was prepared and gel filtered on Superose 12 (see Fig. 1). PEP1 was pooled, dialysed against Mono Q buffer (20 mM Tris,



pH 7.6, 1 mM EGTA, 0.1 mM DTT), loaded onto a Mono Q HR 5/5 column (Pharmacia), and eluted with a gradient from 0 to 1 M KCl in Mono Q buffer. Column fractions were assayed as in Fig. 1 except that for priming assays and PtdIns(4,5)P₂ formation in semi-intact cells (b, f, g), fractions were dialysed into K-Glu buffer and tested in the presence of a limiting dose of PEP3/PtdInsTP to amplify PEP1 activity⁶. Western blotting^{28,29} used affinity-purified anti-type I PtdInsP5K antibodies.

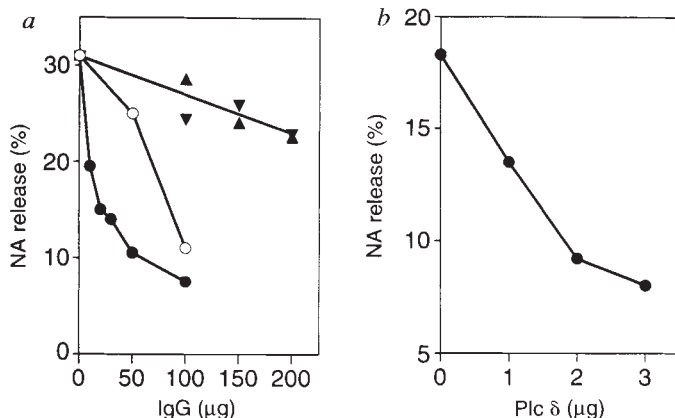


FIG. 4 Inhibition of Ca²⁺-activated noradrenaline secretion by PtdIns(4,5)P₂ antibodies and recombinant phospholipase C-δ. a, Monoclonal PtdIns(4,5)P₂ antibodies (●); Fab fragments (○); control mouse monoclonal antibodies (▲, ▼); b, recombinant phospholipase C-δ.

METHODS. Semi-intact PC12 cells were incubated with 2 mM Mg-ATP and 1.0 mg ml⁻¹ rat brain cytosol for 30 min at 30 °C to prime secretory responsiveness as previously described⁵, and then washed and preincubated on ice with indicated concentrations of antibodies (a) or phospholipase C-δ (b). Free ionic Ca²⁺ was adjusted to 3 μM and either 0.5 mg ml⁻¹ rat brain cytosol (a) or 0.005 mg ml⁻¹ p145/CAPS¹ (b) was added to support Ca²⁺-triggered noradrenaline release detected in three-(a) or one-(b) min incubations at 30 °C. Monoclonal PtdIns(4,5)P₂ antibodies¹⁶ were purified from mouse ascites fluid by chromatography on protein A-Sepharose CL-4B (Pharmacia). Column loading was done in 3M NaCl, 1.5M glycine pH 8.9 and elution in 0.1M sodium citrate pH 3.5. F_{ab} fragments were produced by incubation with agarose-immobilized papain. Antibodies and fragments were exchange into K-Glu buffer before testing. Phospholipase C-δ was produced as a GST fusion protein in *Escherichia coli* using standard methods³⁰. Other GST fusion proteins tested did not inhibit Ca²⁺-triggered [³H]noradrenaline release (not shown).

Hence, the long-recognized requirement for ATP in regulated secretion appears to be, at least in part, for the sequential phosphorylation of PtdIns by a pathway that consists of PEP3/PtdInsTP, a PtdIns 4-kinase and PEP1/PtdInsP5K. The resident PtdIns 4-kinase characterized¹⁸ on dense-core secretory granules may catalyse the second step in this pathway. Our studies do not exclude roles for additional ATP-dependent reactions in the regulated secretory pathway, which may be mediated by NSF¹⁹ and PEP2.

The precise role for PtdIns(4,5)P₂ or a related lipid in Ca²⁺-activated exocytosis remains to be elucidated. A novel role for the intact lipid is likely because phospholipase C inhibited Ca²⁺-activated secretion, and PtdIns(4,5)P₂ metabolites such as inositol trisphosphate, fatty acids and protein kinase C activators did not affect secretion in semi-intact PC12 cells under the Ca²⁺-

buffering conditions used (not shown). PtdIns(4,5)P₂ formation could serve as a signal to recruit proteins to a membrane surface as suggested for the PtdIns(4,5)P₂-binding pleckstrin homology domain contained in fodrin²⁰, which is required for Ca²⁺-regulated secretion²¹. PtdIns(4,5)P₂ synthesis could also promote actin microfilament assembly and membrane-cytoskeletal interactions mediated by the lysine-rich, PtdIns(4,5)P₂-binding domains of cytoskeletal proteins^{22,23}, which may play a role in docking secretory granules at the plasma membrane. Changes in docking following ATP-dependent priming have been inferred from altered neurotoxin susceptibility^{24,25}. Recent studies suggest that PtdIns(4,5)P₂ may also regulate the lipid composition and fusogenic character of membranes as an essential cofactor for the activation of phospholipase D^{26,27}. Recognition of polyphosphoinositides as required cofactors in regulated exocytosis will

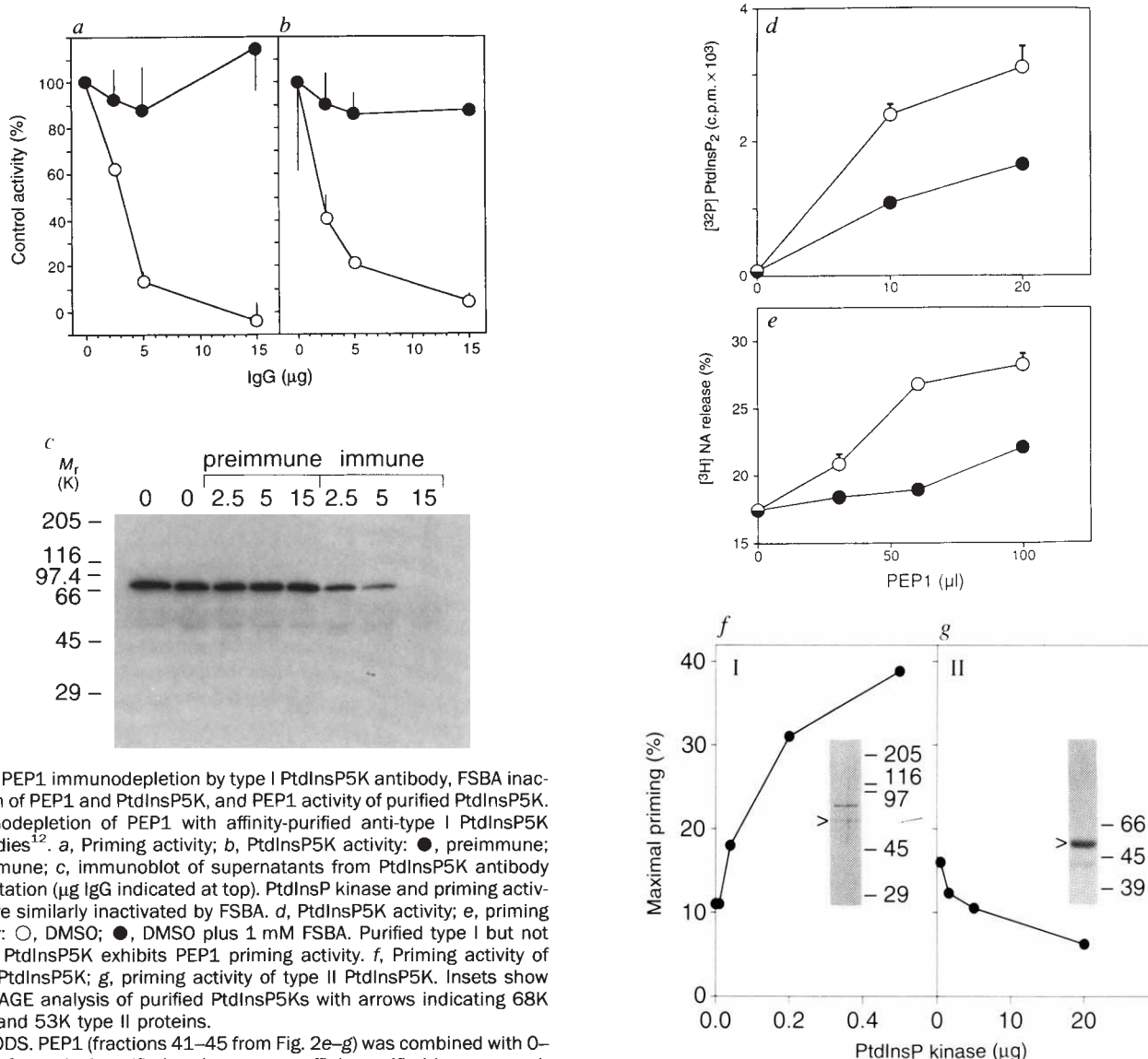


FIG. 3 PEP1 immunodepletion by type I PtdInsP5K antibody, FSBA inactivation of PEP1 and PtdInsP5K, and PEP1 activity of purified PtdInsP5K. Immunodepletion of PEP1 with affinity-purified anti-type I PtdInsP5K antibodies¹². a, Priming activity; b, PtdInsP5K activity: ●, preimmune; ○, immune; c, immunoblot of supernatants from PtdInsP5K antibody precipitation (μg IgG indicated at top). PtdInsP kinase and priming activities are similarly inactivated by FSBA. d, PtdInsP5K activity; e, priming activity: ○, DMSO; ●, DMSO plus 1 mM FSBA. Purified type I but not type II PtdInsP5K exhibits PEP1 priming activity. f, Priming activity of type I PtdInsP5K; g, priming activity of type II PtdInsP5K. Insets show SDS-PAGE analysis of purified PtdInsP5Ks with arrows indicating 68K type I and 53K type II proteins.

METHODS. PEP1 (fractions 41–45 from Fig. 2e–g) was combined with 0–15 μg of protein A-purified preimmune or affinity-purified immune anti-PtdInsP5K antibodies¹² in K–Glu buffer with 0.1% BSA. Following preincubation on ice overnight, IgGSorb protein A reagent (The Enzyme Center) was added for 1 h followed by centrifugation (12,000g for 5 min). Supernatants were tested in priming (with limiting amounts of PEP3/PtdInsTP⁶) and PtdInsP5K assays as in Fig. 1, or electrophoresed and immunoblotted^{28,29} with affinity-purified anti-type I PtdInsP5K antibodies. First lane sample (c) was not treated with IgGSorb. Disappearance of the 90K PtdInsP5K from supernatants was accompanied by a reciprocal appearance in the IgGSorb pellets (data not shown). In control experiments, neither the immune antibody nor the IgGSorb treatment inhibited priming or PtdInsP5K assays. Control activity represents an

IgGSorb-treated supernatant that received no antibody. For FSBA inactivation, a PEP1 fraction (Fig. 1, fractions 30–40) was preincubated for 1 h at 30 °C with 5% DMSO or DMSO plus 1 mM FSBA, and dialysed extensively against K–Glu buffer. FSBA inactivated PtdInsP5K activity half-maximally at 100 μM, similar to that described for PtdIns 4-kinase inactivation¹³. Erythroid type I¹² and type II¹⁴ PtdInsP5K enzymes were purified as previously described. Priming reactions contained a limiting amount of PEP3/PtdInsTP⁶, which was found to potentiate the priming activity of the type I PtdInsP5K. Apparent inhibition of priming by the type II enzyme was not reproducibly observed.

help the elucidation of remaining unknown molecular events in Ca^{2+} -triggered membrane fusion. □

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Activity of RNA polymerase I transcription factor UBF blocked by Rb gene product

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THE protein encoded by the retinoblastoma susceptibility gene (Rb) functions as a tumour suppressor and negative growth regulator¹. As actively growing cells require the ongoing synthesis of ribosomal RNA, we considered that Rb might interact with the ribosomal DNA transcription apparatus. Here we report that (1) there is an accumulation of Rb protein in the nucleoli of differentiated U937 cells which correlates with inhibition of rDNA transcription; (2) addition of Rb to an *in vitro* transcription system inhibits transcription by RNA polymerase I; (3) this inhibition requires a functional Rb pocket²; and (4) Rb specifically inhibits the activity of the RNA polymerase I transcription factor UBF (upstream binding factor) *in vitro*. This last observation was confirmed by affinity chromatography and immunoprecipitation, which demonstrated an interaction between Rb and UBF. These results indicate that there is an additional mechanism by which Rb suppresses cell growth, namely that Rb directly represses transcription of the rRNA genes.

Human monocyte-like U937 cells can be induced to differentiate by the addition of 12-*O*-tetradecanoylphorbol-13-acetate (TPA). Differentiation of U937 cells is accompanied by an accumulation of Rb protein in the nucleoli³, as can be demonstrated by the colocalization of Rb and the nucleolar protein fibrillarin⁴ (Fig. 1). As nucleoli are the site of rDNA transcription, we investigated whether the nucleolar accumulation of Rb correlated with changes in ribosomal RNA synthesis. Nuclear run-on data (Fig. 2a) demonstrated that treatment of U937 cells with TPA resulted in a dramatic decrease in the rate of rRNA synthesis, but did not affect the amount of total cellular RNA during the course of these experiments (data not shown). *In vitro* transcription was performed to test whether Rb itself might repress rDNA transcription. We found that inclusion of a recombinant fusion protein of glutathione-S-transferase with Rb

(GST-Rb)⁵ in an *in vitro* transcription system decreased transcription, whereas inclusion of an inactive Rb protein (GST-Rb209)⁵ had no effect on transcription (Fig. 2b). These results were consistent with a direct interaction between Rb and the rDNA transcription apparatus.

It has been reported that UBF is not required for transcription of rDNA genes but that transcription is stimulated by the presence of UBF^{6,7}. Hence UBF is an auxiliary transcription factor^{6,8}. UBF has been identified as a potential target for Rb⁹. We investigated whether the inclusion of Rb in an *in vitro* transcription reaction affected UBF-independent transcription and/or UBF-dependent stimulation of transcription. The results in Fig. 2c demonstrate that inclusion of either full-length Rb or a biologically active, truncated form¹⁰ of Rb (Rb_T) had no effect on transcription by an extract depleted of UBF. Both forms of Rb repressed activation by UBF, however. In these assays, the high template concentration minimized the effect of UBF⁶. The effect of UBF on transcription was more dramatic when the concentration of template was decreased (Fig. 2d, lanes 1 and 2). The inclusion of GST-Rb in the cell-free transcription assay inhibited the stimulation of transcription by UBF (Fig. 2d, lanes 3 and 4).

Furthermore, inclusion of GST-Rb209 in the assay system had no effect on UBF-stimulated transcription (Fig. 2d, lanes 5 and 6). Repression by GST-Rb could be relieved by including additional UBF in the assay (Fig. 2e). These results indicate that Rb blocked the activation of transcription by UBF, suggesting that an interaction exists between Rb and UBF.

Affinity chromatography experiments were carried out to test this possibility. GST-Rb¹⁰ was bound to glutathione-Sepharose beads and incubated with a nuclear extract from rat Novikoff hepatoma cells. Western blot analysis of the bound proteins demonstrated that UBF bound to GST-Rb, but not to GST-Rb209 or glutathione-Sepharose alone (Fig. 3a).

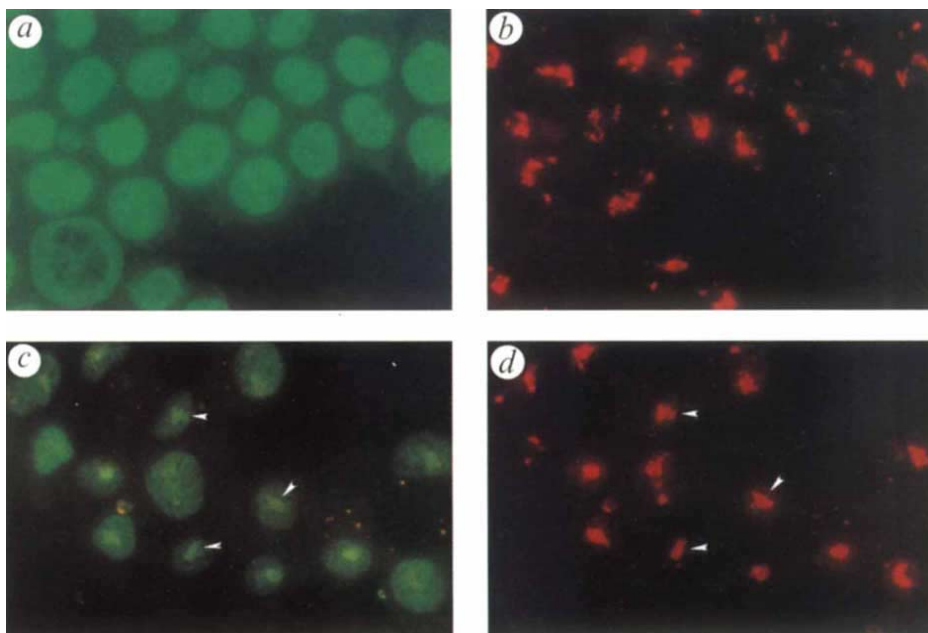
Viral and cellular proteins that interact with the Rb pocket contain the consensus sequence LxCxE^{2,11}. Such a consensus sequence is found in the third HMG box of rat UBF⁹. As shown in Fig. 3b, the inclusion of the peptide HPV-16 E7, containing a functional LxCxE consensus², blocked the binding of UBF to GST-Rb (lanes 5–8), whereas an inactive form of the E-7 peptide² (E-7*) had no effect (Fig. 3b, lanes 1–4). Moreover, the inclusion of E-7, but not E-7*, blocked the inhibitory effect of Rb on rDNA transcription (data not shown). These experiments demonstrated that Rb and UBF could interact through the Rb pocket, thereby affecting the ability of UBF to activate transcription.

We also determined whether an interaction between Rb and UBF could be detected in cell extracts. Nuclear extracts from LTR α 3 cells were incubated with anti-UBF antibody bound to

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FIG. 1 Rb translocates to the nucleoli of U937 cells upon TPA induced differentiation. Colocalization of Rb (a, c) and fibrillarin (b, d) in control (a, b) and TPA-treated (10^{-7} M) U937 cells (c, d).

METHODS. Cytosin smears of suspended, control U937 cells or differentiated cells, removed by shaking, were fixed in methanol at 10°C for 10 min. Rb protein was stained by incubating the smears with anti-Rb antibody (AB-4, Oncogene Science) at a final concentration of $25\text{ }\mu\text{g ml}^{-1}$. Mouse IgG (PharMingen) at the same concentration was used as a negative control. The smears were treated with affinity-purified fluorescein-conjugated sheep anti-mouse antibody at a final dilution of 1:250 (Organon Technika). Nucleoli were visualized with human anti-fibrillarin antibody (Sigma) and then with affinity-purified goat anti-human rhodamine-conjugated antibody (Organon Technika) at a final dilution of 1:100. The smears were incubated with both primary antibodies simultaneously and then with a mixture of the anti-mouse and anti-human secondary antibodies. Both incubations were performed at 37°C for 30 min. After each incubation, the cells were washed (10 min $\times$ 3) with saline buffered with 10 mM Tris-HCl, pH 7.3.



Slides were mounted in vectrashield anti-bleaching medium (Vector), viewed under a Zeitz (Lablux 12) microscope at a final magnification of 1,000. Similar results were obtained with another mouse monoclonal anti-Rb antibody (PharMingen).

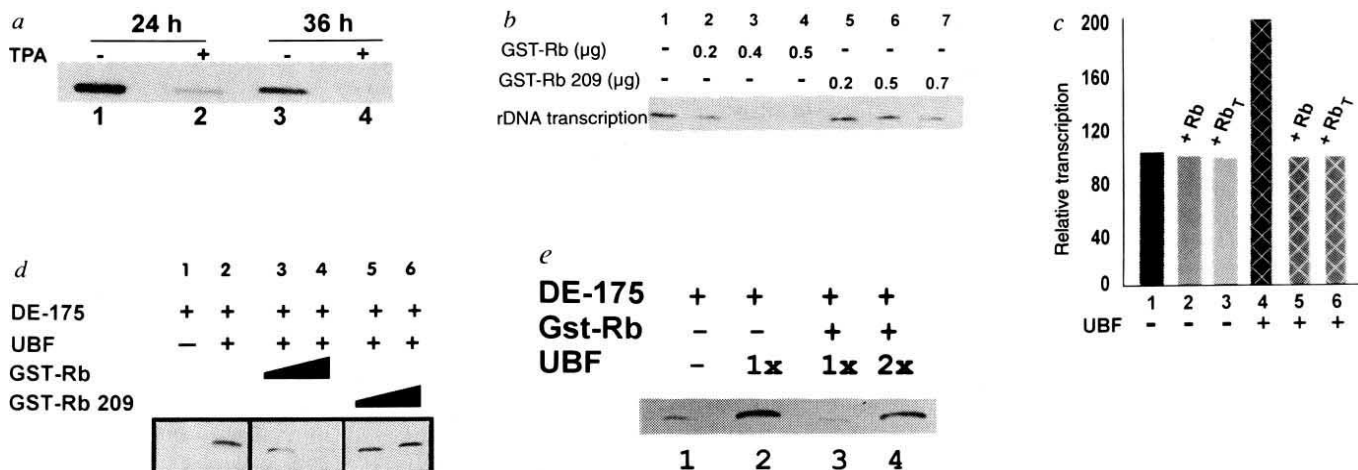


FIG. 2 Effect of Rb on ribosomal DNA transcription. **a**, Nuclear run-on analysis of rRNA synthesis by U-937 cells treated for 24 or 36 h with 10^{-7} M TPA. Nuclei, isolated from either control or TPA-treated U-937 cells, were incubated in nuclear run-on assays¹⁷, and rDNA transcription determined by hybridizing the *in vitro* synthesized RNA to the 5' end of human rDNA gene immobilized on nitrocellulose. **b**, Rb can inhibit rDNA transcription *in vitro* at low template concentrations; **c**, Rb has no effect on basal transcription by RNA polymerase I at high template concentrations but inhibits activation by UBF at low template concentrations (**d**); **e**, the presence of additional UBF can overcome the effect of Rb on UBF-dependent transcription.

METHODS. *In vitro* transcription reactions, containing Novikoff hepatoma cell nuclear extracts, 0.002 μg (**b**), 0.2 μg (**c**), or 0.02 μg (**d**, **e**) of *Eco*RI-linearized template, p5.1E/X, and 1.0 μg nonspecific DNA were performed and analysed as described^{6,8}. GST-Rb fusion proteins containing amino acids 394–928 of the retinoblastoma protein fused to the carboxy-terminal region of glutathione-S-transferase in pGEX-2T (Pharmacia)¹⁰ were purified by chromatography on glutathione-

Sepharose (Pharmacia). GST-Rb 209 contains an amino-acid substitution of phenylalanine for cysteine at position 706 (ref. 5). Novikoff hepatoma ascites cell nuclear extracts were fractionated by DEAE-Sephadex column chromatography⁶, resulting in a fraction, DE-175, which is essentially devoid of UBF^{6,8}. UBF was purified through the CM-Sephadex column chromatography step⁶. Transcription by the UBF-depleted fraction was set at 100% in the graph. The Rb protein used in **c** was the full-length 110K protein and Rb_T was an N-terminal truncation product of Rb containing amino acids 394–928. In **d**, GST-Rb (lanes 3 and 4) and GST-Rb209 (lanes 5 and 6) were added at 1x (lanes 3 and 5) and 3x (lanes 4 and 6) the molar concentration of UBF, as indicated by the wedges. The levels of transcription relative to lane 2 were (by lane): 0.2, 1.0, 0.6, 0.3, 0.9 and 10, respectively. In **e**, UBF was present at 1x and 2x the molar concentration of Rb, as indicated.

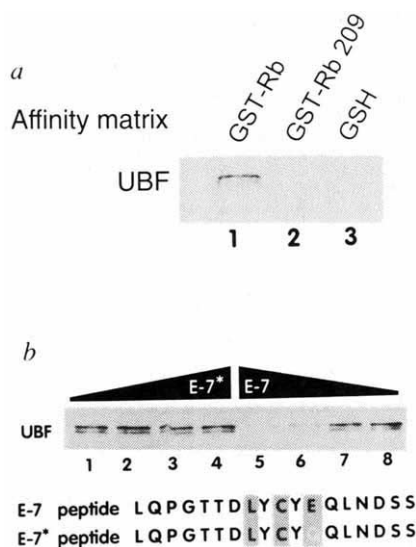


FIG. 3 Interaction between UBF and Rb. **a**, UBF binds to wild-type Rb but not to RB209.

METHODS. *E. coli* lysates, prepared as described⁹, containing GST-Rb or GST-Rb209, were incubated, separately, with glutathione-Sepharose beads (Pharmacia) for 1 h at 4 °C. The beads were washed twice with 200 volumes of 20 mM Tris, pH 8.0, 100 mM NaCl, 1 mM EDTA, 0.5% NP-40 (NETN), and then twice with PBS. Nuclear extracts from rat Novikoff hepatoma ascites cells, containing authentic UBF, were incubated with the immobilized forms of Rb for 1 h at 4 °C. The beads were washed four times with 200 volumes of NETN, and then an equal volume of 2 × SDS sample buffer was added. After boiling, samples were fractionated by SDS-PAGE on 10% acrylamide gels, blotted onto nitrocellulose, and UBF that had bound to the beads was detected with a monospecific antisera that recognizes both the 97K and 94K forms of UBF⁴. Western analysis was detected by ECL (Amersham). **b**, UBF interacts with the Rb pocket. Nuclear extracts were incubated with GST-Rb beads as in **a**, except that either E-7 or E-7* peptide² was added to the nuclear extract before it was incubated with immobilized Rb. 0.1, 1.0, 10 or 20 µg of either peptide, as indicated by the wedges, was included in each incubation. Sequences of the two peptides are shown.

protein A-agarose. Proteins that bound to the antibody-affinity beads were analysed by sequential western blot analysis (Fig. 4a), which indicated an association of Rb with protein immunoprecipitated by the antibody specific for UBF (lanes 3 and 4). Rb was not detected in immunoprecipitates when the E-7 peptide was preincubated with cell extract (lanes 5 and 6). Thus, an Rb-UBF complex exists in cell extracts.

Results depicted in Fig. 4b represent immunoprecipitation experiments employing extracts of control and TPA-treated U937 cells. Concomitant with the accumulation of Rb in the nucleoli of U937 cells, there was an increase in the amount of Rb protein that coimmunoprecipitated with UBF, demonstrating an increased association of UBF and Rb in TPA-treated cells. Western blot analysis of total cell extracts ruled out the possibility that the increased association of UBF and Rb was due to an increase in the amount of Rb or UBF protein during differentia-

tion (Fig. 4c). It has been suggested that Rb plays a role in the differentiation of myoblasts¹². Further studies on the role of Rb in the differentiation of haemopoietic cells may reveal roles for Rb in this differentiation pathway¹³.

The activity of UBF may be regulated by phosphorylation^{4,8} or by regulation of its expression¹⁴, and, as suggested by these results, a third mechanism in which Rb sequesters UBF activity. In preliminary experiments, recombinant Rb bound recombinant UBF (data not shown), suggesting that the interaction between the two proteins is direct. As UBF and RNA polymerase I may also interact¹⁵, the binding of Rb to UBF may block this interaction and the subsequent UBF-dependent activation of transcription. Rb protein has been shown to associate with various proteins involved in cell proliferation¹⁶. This is the first report to implicate Rb in an interaction with the RNA polymerase I transcription system. As rDNA transcription is required

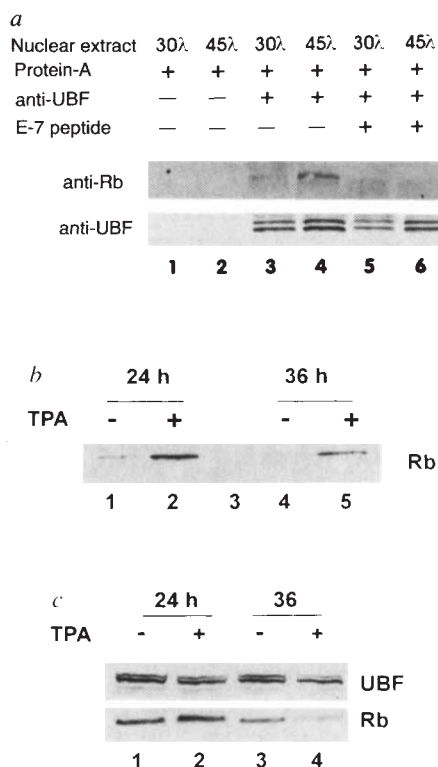


FIG. 4 Rb can be coimmunoprecipitated with UBF. **a**, Nuclear extract from LTRα3 cells (30 or 45 µl) was incubated with protein A-agarose (lanes 1 and 2), anti-UBF coupled protein A-agarose (lanes 3 and 4), or anti-UBF coupled protein A-agarose plus E-7 peptide (lanes 5 and 6). The resulting immunoprecipitates were resolved by SDS-PAGE and subjected to sequential western blot analysis using antibodies specific for Rb and UBF. **b**, Differentiation of U937 cells upon TPA treatment (10⁻⁷ M) increases the association of UBF with Rb. **c**, Treatment with TPA does not increase UBF or Rb protein.

METHODS. **a**, Nuclear extract was diluted 1:1 with buffer C/20 (ref. 6) and precleared by mixing with protein A-agarose (Sigma) for 60 min at 4 °C followed by a brief centrifugation. 60 or 90 µl of precleared extract was applied to protein A-agarose or anti-UBF-coupled protein A-agarose beads, adjusted to 200 µl with buffer C containing 10% glycerol (C/10), and brought to 0.2% NP-40. When added, the E-7 peptide (40 µg), was incubated with 200 µl of precleared extract for 30 min at 4 °C before being mixed with the anti-UBF-protein A-agarose beads. All samples were incubated for 3 h at 4 °C. The beads were washed twice with buffer C/10 containing 0.2% NP-40 and resuspended in 2 × SDS-PAGE sample buffer. Immunoprecipitated proteins were resolved by SDS-PAGE (10% polyacrylamide), transferred to nitrocellulose (Biorad Laboratories), and probed sequentially with a monoclonal anti-Rb antibody (Oncogene Sciences) and with anti-UBF antibody to assess the efficiency of immunoprecipitation of UBF from the extracts. **b**, Control cells (lanes 1 and 4) or cells treated with TPA (lanes 2 and 5) for 24 or 36 h were immunoprecipitated with an anti-UBF antibody as described. Lane 3 represents immunoprecipitation with protein A-Sepharose alone. **c**, Cell lysates prepared from both control (lanes 1 and 3) and cells treated with TPA for 24 or 36 h (lanes 2 and 4), were fractionated by SDS-PAGE, blotted onto nitrocellulose and probed sequentially with antibodies against Rb and UBF. Western analysis was detected by ECL.

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cleavage products of RNA I are differentially susceptible to PNPase digestion despite their identical sequence. That the extent of phosphorylation at the 5' end can in fact affect the activity of PNPase was demonstrated directly by treating pppRNA I₋₅ with tobacco acid-pyrophosphatase, which removes a pyrophosphate group from the 5' end and produces a 5' monophosphate terminus. The pyrophosphate-treated substrate was degraded by PNPase *in vitro* at 2–3 times the rate observed for pppRNA I₋₅ (Fig. 3b, c).

Our failure to detect an increased fraction of polyadenylated RNA I derivatives in *pnp* mutant bacteria suggests that 3' aden-

osine residues can also be removed from RNA I by another enzyme, perhaps RNase II, which, like PNPase, has 3' to 5' exoribonucleolytic activity¹⁹. However, the postulated digestion by a second enzyme does not continue at a rapid rate through primary transcripts, as degradation of RNA I is retarded in cells mutated in *pnp* (Fig. 2). Molecules containing only a few adenosine residues at their 3' termini (those species slightly longer than the full-length primary transcript) decayed at the same rate as the corresponding non-adenylated RNA. Additionally, in the absence of post-transcriptional adenylation by the *pcnB*-encoded poly(A) polymerase¹³, an RNA I variant that had

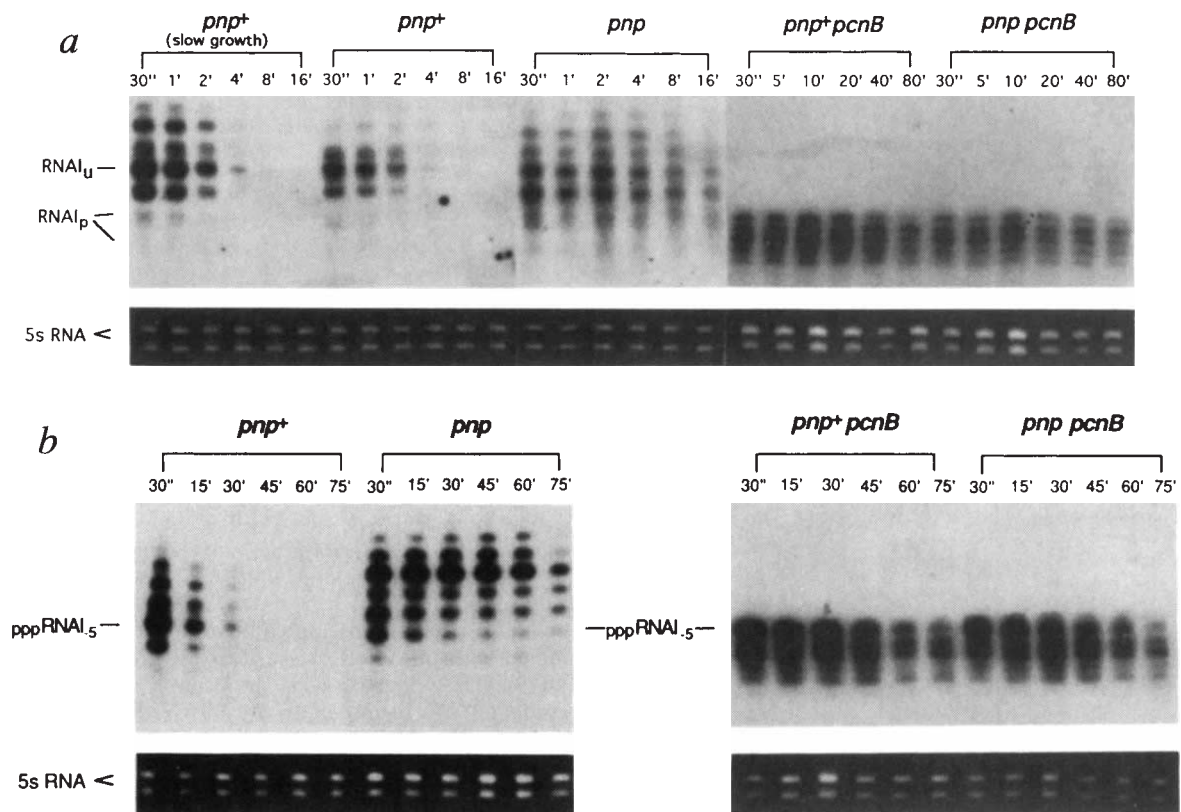
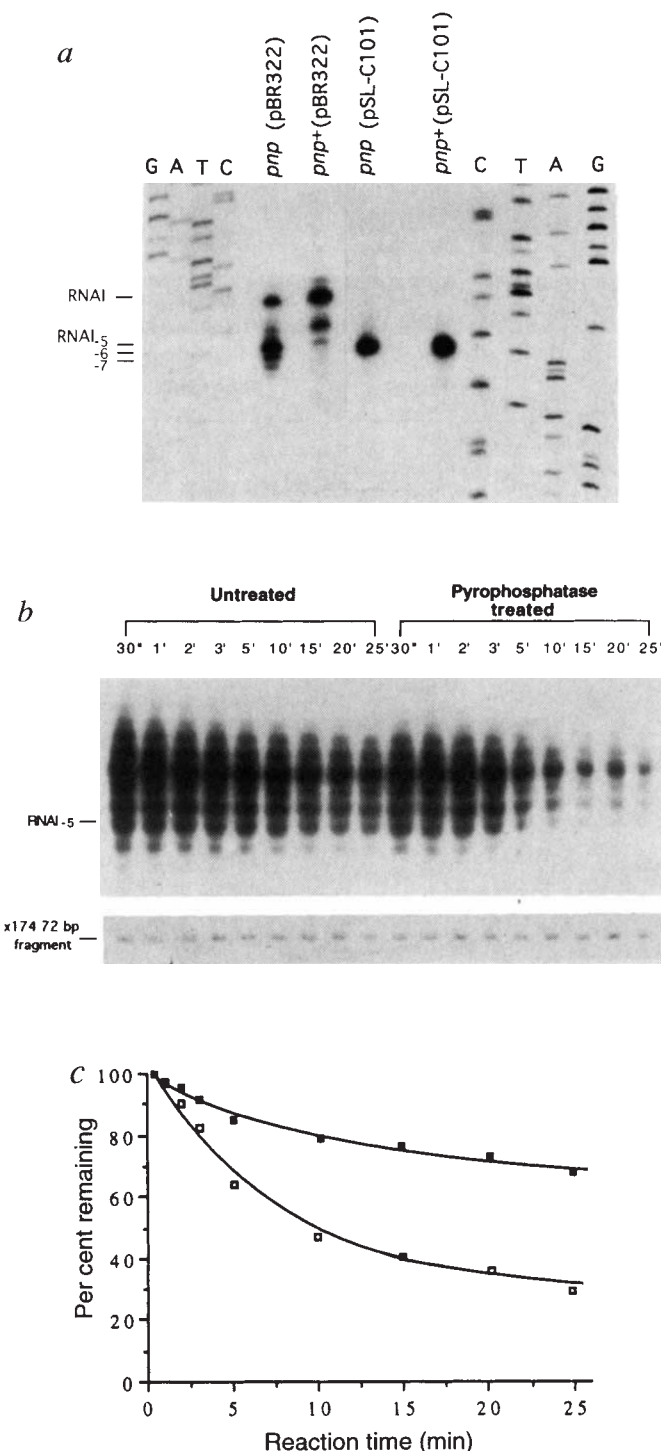


FIG. 2 Degradation of RNA I by PNPase. Decay of pBR322 RNA I (a) and pSL-C101 pppRNA I₋₅ (b) in *pnp*⁺ (CA244), *pnp* (CA244, *pnp*:Tn5)²⁸, *pnp*⁺ *pcnB* (Mri93, ref. 12), and *pnp* *pcnB* (constructed by P1 transduction of the *pnp* mutation to Mri93) *E. coli* strains. Decay of RNA I in *pnp*⁺ bacteria was also measured at the slow growth rate of *pnp* mutants. Positions of primary transcripts RNA I_U and pppRNA I₋₅, and of RNase E cleavage products (RNA I_P), were identified previously using unprocessed RNA I (108 nt) and pppRNA I₋₅ (103 nt) transcripts made *in vitro* as size standards⁸. Species longer than primary transcripts are 3' adenylated⁸. Table 1 shows the results of densitometric quantitation of relevant RNA half-lives. c, PNPase digestion *in vitro*. pRNA I₋₅, circles; pppGGA-RNA I, squares; white and black symbols indicate non-adenylated and adenylated RNA, respectively. No degradation was observed when KCl was substituted for K₂HPO₄ in the assay buffer. The data shown reflect the combined effects of PNPase insensitivity of some molecules and rapid decay of others (compare with refs 16, 17). METHODS. Northern blot analysis^{8,11} which used RNA (absorbance at 260 nm (*A*₂₆₀) units ~0.5) isolated after the addition of rifampicin, resolved RNA species differing by one nucleotide in length. 5S RNA was used as an internal loading standard to quantify RNA bands detected by a RNA II riboprobe^{8,11}. ³²P-labelled pppGGA-RNA I transcripts²⁷ (8,500 c.p.m. 0.1 pmol) were denatured in water, adenylated by incubation for 15 min at 25 °C with 0.35 U *E. coli* poly(A) polymerase (PcnB protein¹³) (Life Technologies, Gaithersburg, MD) in 50 µl reaction buffer as described by the manufacturer, and extracted with phenol. The adenylated RNA (0 to 200 3' adenosines long, data not shown; compare with ref. 29) was denatured in water, renatured in 70 µl of 1.07 × PNPase digestion buffer²³ for 30 min at 37 °C, and digested by

addition of 5 µl (0.15 U) *E. coli* PNPase (Panvera, Madison, WI). Non-adenylated RNA was treated in parallel except that poly(A) polymerase was omitted from the first incubation. pRNA I₋₅ was prepared by gel purification of RNase E¹⁸-treated pppGGA-RNA I. The figure shows the percentage of undegraded RNA retained on Whatman GF/C paper after TCA precipitation of 10-µl samples taken at the indicated time points.

six adenosines added mutationally to its 3' end during transcription *in vivo* also did not show accelerated decay (data not shown). These observations, plus evidence that the only adenylated RNA I species detected in bacteria containing a functional *pnp* gene had short poly(A) tails (Fig. 2a, b; and ref. 8), support the notion that RNA I molecules containing longer poly(A) tails are the ones attacked by PNPase.

Regions of secondary structure can protect upstream RNA segments from rapid decay *in vivo*²⁰⁻²³ and impede 3' to 5' exonucleolytic digestion by PNPase *in vitro*²³. However, despite the presence of three stable stem-loop regions in RNA I and the strikingly tRNA-like overall conformation of this RNA (Fig.



Strain and plasmid	RNA I species	Half-life (min)
pBR322		
<i>pnp</i> ⁺	RNA I ₀	1.4 ± 0.1*
	RNA I _p	<1.4
<i>pnp</i>	RNA I ₀	8.4 ± 1.0
	RNA I _p	12.0 ± 1.2
<i>pnp</i> ⁺ <i>pcnB</i>	RNA I _p	>60
<i>pnp</i> <i>pcnB</i>	RNA I _p	>60
pSL-C101		
<i>pnp</i> ⁺	pppRNA I ₅	7.8 ± 0.5*
<i>pnp</i>	pppRNA I ₅	13.4 ± 0.8
<i>pnp</i> ⁺ <i>pcnB</i>	pppRNA I ₅	>60*
<i>pnp</i> <i>pcnB</i>	pppRNA I ₅	>60

RNA I half-life was determined as described after densitometric scanning of autoradiographs of gels⁴¹. The $t_{1/2}$ shown for RNA I₀ represents the value determined for pBR322 RNA I transcripts 106 to 111 nt in length, all of which decay at a similar rate (see text and ref. 8). The value shown for RNA I_p indicates the collective half-life for the products of RNase E cleavage of pBR322 RNA I (see text, Fig. 2 and ref. 8). pppRNA I₅ is a mutationally truncated RNA I derivative encoded by the pSL-C101 plasmid (see text and ref. 11). Data identified with * have been published previously⁸ and were confirmed in this study. The half-life reported here for pBR322 RNA I_p in a *pcnB* host, which was determined under sampling conditions that allowed measurements over an extended period of time, is longer than the one reported earlier⁸.

1), RNA I normally does not show the long half-life typical of highly structured molecules such as tRNA and ribosomal RNA¹⁰. Although decay of RNA I and at least one species of ribosomal RNA (9S rRNA) is initiated by RNase E²⁴, which cleaves both RNAs *in vitro* with roughly the same efficiency¹⁸, the 9S rRNA cleavage products are stable whereas the products of RNase E cleavage of RNA I are rapidly degraded *in vivo*. The results reported here indicate that the combined actions of poly(A) polymerase and PNPase have a prominent role in regulating half-life of the RNase E-generated intermediates of RNA I, and that the action of PNPase is influenced by events at both the 5' and 3' ends of RNA I. We speculate that susceptibility of RNA to polyadenylation-mediated 3' to 5' digestion by PNPase is a key factor in determining more generally whether transcripts will show the short half-life of mRNA or

FIG. 3 Functional interaction of ribonucleases acting at the separate ends of RNA I. **a**, Primer extension analysis⁸ of the effect of *pnp* mutation on pBR322-encoded RNA I and pSL-C101-encoded RNA I₅. pBR322-encoded bands 5, 6 and 7 nt from the 5' end of primary transcripts represent species generated by or following RNase E cleavage⁸ (RNAI_p, see Table 1), whereas the band at position 3 is *rne/ams* independent¹⁸. **b**, Effect of 5' phosphorylation on PNPase-mediated decay. The species banding at various positions were identified previously⁸ (see also Fig. 2 legend). **c**, Plot of decay of ppp (black squares) and pRNA I₅ (white squares) in **b**.

METHODS. pSL-C101 encoded pppRNA I₅ was isolated from the *pnp* mutant strain and purified by filter-hybridization⁸. Treatment of pppRNA I₅ with tobacco acid pyrophosphatase according to the vendor's instructions (Epicentre Technologies, Madison, WI) generated pRNA I₅. RNA samples (50 pmol) were treated as in Fig. 2c before PNPase digestion. Reaction mixtures (50 µl) contained 0.15 U PNPase plus 0.5 µg µl⁻¹ φX174 RF form DNA/HaeIII DNA fragments that were used as an internal standard and probed with ³²P-labelled φX174 DNA. At the indicated times, 8-µl volumes were removed from reaction mixtures and added to 8 µl of loading dye solution containing 98% formide, 0.03% bromophenol blue and 0.03% xylene cyanol. Northern blot analysis done as described in Fig. 2a and b measures the disappearance of full length RNA₅, whereas TCA precipitation, as shown in Fig. 2c, measures decay intermediates that remain acid-insoluble; when degradation of the substrates shown in 2c was analysed by gel electrophoresis (data not shown), the decay rate was similar to that seen in c.

the stability of tRNA and rRNAs. Thus, the normally rapid turnover of mRNA in *E. coli* may require adenylation to aid 3' exonucleolytic digestion through an otherwise resistant conformation, as we have observed for RNA I. Recent evidence that decay of several different mRNAs is impeded in *pcnB* mutants^{25,26} is consistent with this notion. □

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A structural basis of the interactions between leucine-rich repeats and protein ligands

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THE leucine-rich repeat is a recently characterized structural motif¹ used in molecular recognition processes as diverse as signal transduction, cell adhesion, cell development, DNA repair and RNA processing². We present here the crystal structure at 2.5 Å resolution of the complex between ribonuclease A and ribonuclease inhibitor, a protein built entirely of leucine-rich repeats. The unusual non-globular structure of ribonuclease inhibitor, its solvent-exposed parallel β -sheet and the conformational flexibility of the structure are used in the interaction; they appear to be the principal reasons for the effectiveness of leucine-rich repeats as protein-binding motifs. The structure can serve as a model for the interactions of other proteins containing leucine-rich repeats with their ligands.

The crystal structure of the complex between bovine ribonuclease (RNase) A and porcine ribonuclease inhibitor (RI) was determined with the method of molecular replacement. The atomic model was refined at 2.5 Å resolution to an *R*-value of 19.4%. Structure solution and model refinement are summarized in Table 1.

The primary structure of RI is built of 15 leucine-rich repeats (LRRs), alternately 28 and 29 residues long³. In three dimensions, individual LRRs represent structural β - α hairpin units³. The β -strand and the α -helix are roughly parallel in individual β - α units, and the units are all aligned roughly parallel to a common axis. The resulting structure has a non-globular shape resembling a horseshoe. A long parallel β -sheet lines the inner circumference of the horseshoe and has its concave face exposed to the solvent.

RNase A is kidney-shaped with the active site located in a deep cleft^{4,5}. It is predominantly built of antiparallel β -structure with a prominent N-terminal helix making contacts to each of the two lobes. RNase A binds to the inhibitor with its active site cleft centred on the C-terminal corner of the horseshoe; one lobe of RNase A reaches into the concave cavity of the horseshoe, and the other lobe covers the face of the horseshoe (Fig. 1). RNase activity is inhibited by preventing access of a substrate to the active site. Several RNase A residues involved in binding substrate analogues instead interact with RI in the RNase A–RI complex^{7–9}. The contacts between RNase and RI are summarized in Table 2.

With 456 residues, the inhibitor is much larger than RNase A with 124 residues. By binding RNase A to a concave region of its surface, RI uses its larger size to provide an extensive binding area for the RNase; 2,551 Å² of accessible surface is buried in the interface from a 1.4 Å probe (program SURFACE¹⁰). This is 60% more than typical for protease-inhibitor and antibody–protein antigen complexes¹¹ and is probably one of the main reasons for the tightness of the association ($K_i = 5.9 \times 10^{-14}$ M (ref. 12)). The interface is 49% non-polar, 27% polar and 24% charged; most protease-inhibitor and antibody–protein antigen recognition sites are less charged (typically 55% non-polar, 25% polar and 20% charged¹¹). All 30 free sulphhydryl groups of RI and the four disulphide bridges of RNase A remain intact in the complex. The observed disruption of the interaction by thiol reagents¹³ is probably due to their effect on the structure of RI¹⁴.

The bulk of the interaction concentrates at the C-terminal part of the inhibitor (Fig. 1d). Out of 28 residues that form close contacts with RNase A with distances below 4 Å, 9 are in the β -sheet (contributed by 7 different β -strands), only 2 belong to α -helices, and the remaining 17 come from the loops connecting C termini of β -strands with N termini of helices. Although the β -sheet contains only 11% of all RI residues, it contributes 32% of interacting residues.

The structure of RNase A in the complex does not substantially differ from the structure of free RNase A¹⁵ (the r.m.s. deviation of the Ca atoms after superposition is 0.54 Å); the conformation of RI, however, is different in the crystals of free RI⁴ and the crystals of RNase A–RI complex. RI accommodates the RNase molecule by enlarging the cavity in the structure; the shortest distance between the N-terminal and C-terminal repeats of RI increases from about 12 Å in the free form to over 14 Å in the complex. The r.m.s. deviation of the superimposed Ca atoms of the free and bound forms is 1.46 Å. Unlike most other

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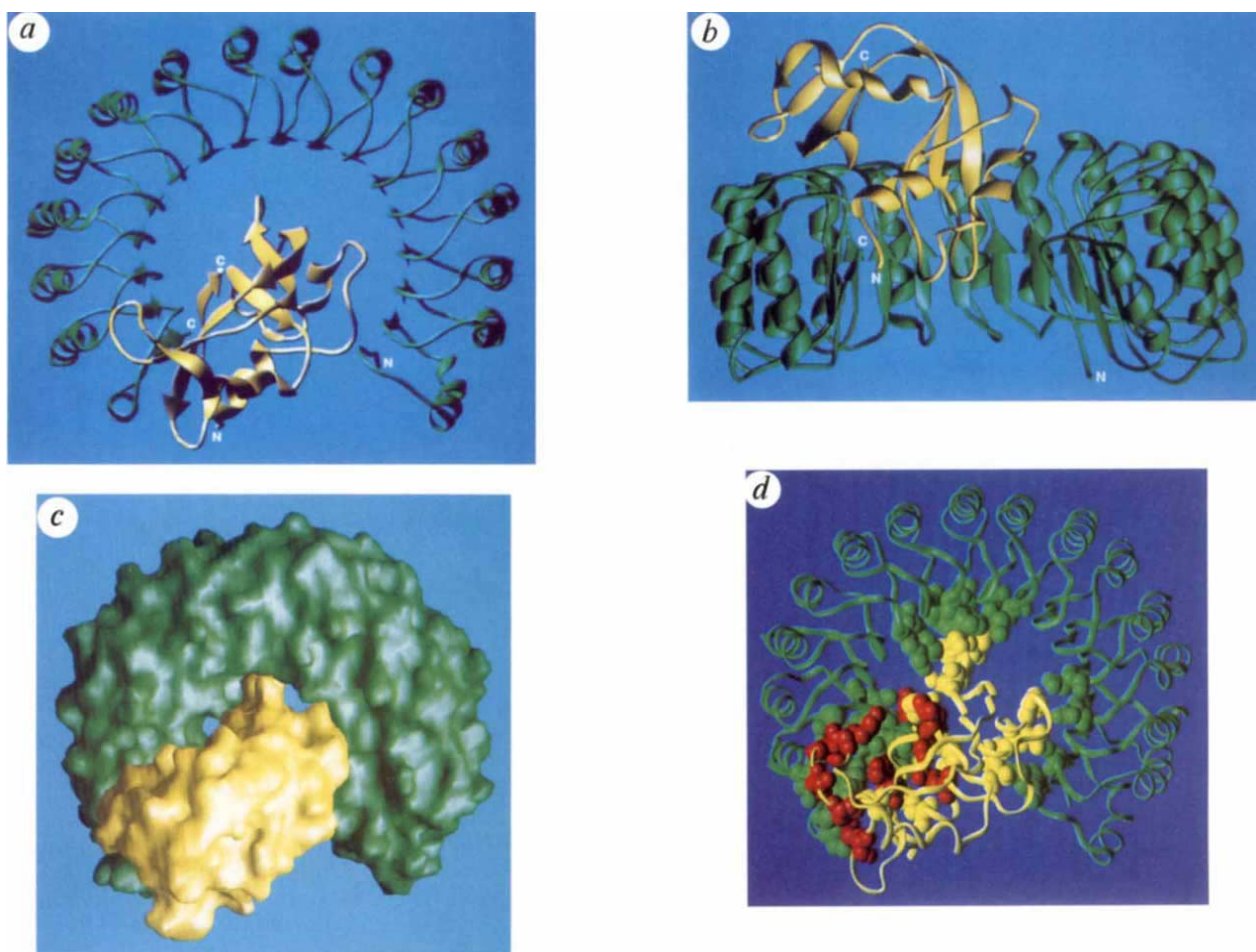


FIG. 1 Structure of RNase A-RI complex. RI is green and RNase A is yellow. *a*, Schematic diagram of the three-dimensional structure of RNase A-RI complex, drawn with the program RIBBONS²⁸. The N and C termini of both molecules are indicated. *b*, Same as in *a*, but rotated around the horizontal axis for about 90°. *c*, Molecular surface of the RNase A-RI complex, drawn with the program GRASP²⁹. The orientation

is similar to that in *a*. *d*, The RNase A-RI interface. The residues involved in intermolecular contacts at a distance below 4 Å (Table 2) are shown as space-filling models. The rest of the structure is shown as a ribbon diagram. The RNA-binding residues of RNase A that contact RI in the RNase A-RI complex (Table 2) are shown in red. The figure was prepared with the program INSIGHT (Biosym). The orientation is similar to that in *a*.

FIG. 2 Conformational changes in RI upon binding RNase A. The models were superimposed with the program INSIGHT (Biosym) using only C α atoms. *a*, r.m.s. deviations of the C α atoms of the models of RI in the crystals of free RI and the crystals of RNase A-RI complex. The dashed line represents a fourth-order polynomial curve fitted to the r.m.s. deviations curve. *b*, Superposition of the models of RI in the crystals of free RI and the crystals of RNase A-RI complex. In the stereo diagram, only the C α traces are shown. The model of free RI is blue and the model of RI from the RNase A-RI complex is green. The figure was drawn with the program MOLSCRIPT³⁰.

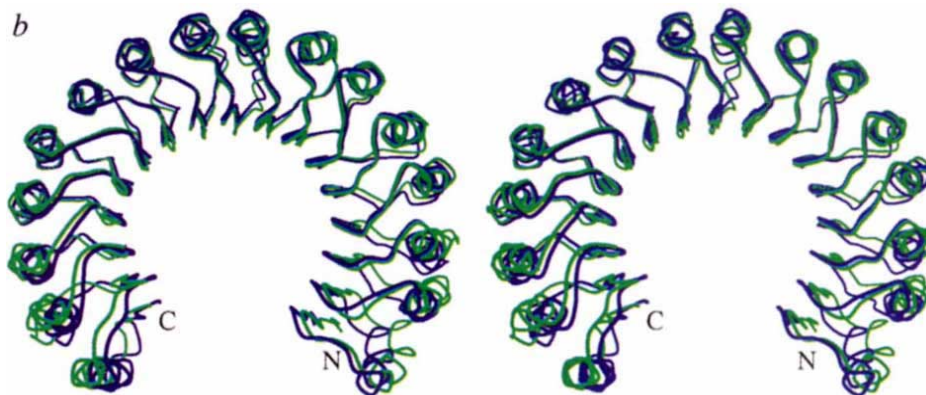


TABLE 1 Refinement statistics

Resolution (Å)	40–2.5
Number of reflections used ($F > \sigma(F)$)	18,859
R_{cryst}^* (%)	19.4
Total number of non-hydrogen atoms	4,416
Number of non-hydrogen protein atoms	4,365
Number of putative water molecules	46
Number of putative sulphate molecules	1
Average B -factors (Å ²)	
RNase inhibitor: all	46.7
main chain	43.3
side chain	50.6
RNase A: all	58.6
main chain	58.2
side chain	59.0
r.m.s. bond length deviation (Å)	0.013
r.m.s. bond angle deviation (°)	1.826

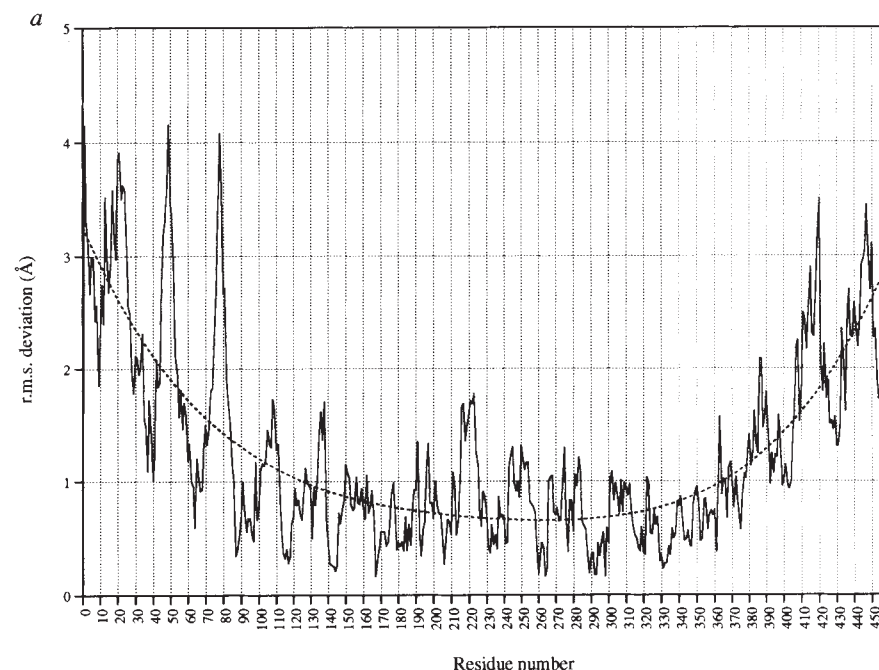
RI was purified from porcine liver²⁰; RNase A from bovine pancreas was purchased from Sigma (type XII-A). Crystals of the RNase A-RI complex have the symmetry of the tetragonal space group $I4$ with $a = 133.3$ Å and $c = 86.7$ Å (ref. 21). Two crystals were used to collect oscillation images at room temperature on an R -axis II image plate detector (Molecular Structure Corporation) using $\text{CuK}\alpha$ radiation from a Rigaku rotating anode generator. The images were processed with the programs DENZO and SCALEPACK²². A total of 111,046 observations were measured and subsequently reduced to 26,431 unique reflections with $R_{\text{merge}} = 0.100$ for $I > -3\sigma(I)$ ($R_{\text{merge}} = \sum_{hkl} (\sum_i (|I_{hkl,i}| - \langle I_{hkl} \rangle)) / \sum_{hkl,i} \langle I_{hkl} \rangle$, where $I_{hkl,i}$ is the intensity of an individual measurement of the reflection with Miller indices h, k and l , and $\langle I_{hkl} \rangle$ is the mean intensity of that reflection). Of the theoretically observable reflections, 93.2% were measured between 40 and 2.5 Å resolution, 71.4% of the measured reflections had $F > \sigma(F)$. Version 3.1 of the program package X-PLOR²³ was used for phase determination and refinement; the program package O²⁴ was used for inspection of atomic models and electron density maps and for model building. The starting phases of the RNase A-RI crystals were obtained by positioning the atomic model of porcine RI⁴

(entry 1BNH in Protein Data Bank) in the crystals of RNase A-RI complex by the method of molecular replacement. The orientation of RNase A could not be found in crossrotation functions, but its location was revealed in electron density maps based on the properly positioned RI molecule; the model of RNase A¹⁵ (entry 7RSA in Protein Data Bank) was positioned into this electron density on the graphics display. The resulting model containing both RI and RNase A had an R_{cryst} of 49.0% using data between 10 and 3.5 Å resolution and $F > 2\sigma(F)$. The inhibitor and the enzyme were refined as rigid bodies resulting in an R_{cryst} of 42.3% and $R_{\text{t}}^{\text{free}}$ of 43.0% between 10 and 3.5 Å resolution and $F > 2\sigma(F)$ ($R_{\text{t}}^{\text{free}}$ is equivalent to R_{cryst} but calculated with 10% of reflections omitted from the refinement process²⁵). Further refinement, dividing the inhibitor in two, four and eight rigid bodies of roughly equal size, resulted in an R_{cryst} of 33.3% and $R_{\text{t}}^{\text{free}}$ of 34.2% for data between 10 and 3.5 Å resolution and $F > 2\sigma(F)$. Cycles of positional refinement, refinement of individual isotropic temperature factors, manual rebuilding, and addition of solvent molecules, reduced R_{cryst} to 19.4% and $R_{\text{t}}^{\text{free}}$ to 28.6% using data with $F > \sigma(F)$ between 40 and 2.5 Å resolution and bulk solvent correction. Peaks in the electron density map calculated with model-derived phases and coefficients $|F_{\text{obs}}| - |F_{\text{calc}}|$ were selected as candidate water molecules with the program WATER (written by S. R. Sprang); only waters within 3.5 Å of a polar atom and B -factors below 80 Å² were kept in the model. In the final stage of refinement we included the data omitted from refinement for calculation of $R_{\text{t}}^{\text{free}}$, which resulted in an improvement of the electron density maps. The final model contains all 456 RI residues including the N -acetyl group, all 124 residues of RNase A, 46 water molecules, and one sulphate molecule. All non-glycine residues have Φ, Ψ angles within allowed hard-sphere limits. The mean coordinate error is estimated to be 0.32 Å based on the Luzzati plot²⁶ and 0.57 Å based on the SIGMA method²⁷. For reasons we currently do not understand, the models of RNase A and RI have average B -factors higher than is usually observed in crystal structures at a comparable resolution. Nevertheless, the final electron density was of high quality and contained no regions of discontinuous density in the backbone of the protein when contoured at 1σ . The coordinates will be deposited in the Brookhaven Protein Data Bank. More details of the structure determination and the mechanism of ribonuclease inhibition will be presented elsewhere.

* $R_{\text{cryst}} = \sum_{hkl} (||F_{\text{obs}}| - |F_{\text{calc}}||) / \sum_{hkl} |F_{\text{obs}}|$, where $|F_{\text{obs}}|$ and $|F_{\text{calc}}|$ are the observed and calculated structure factor amplitudes.

conformational changes observed in proteins, there is no obvious hinge involved; small shifts accumulate along the chain, resulting in changes of the curvature and the twist of the horseshoe (Fig. 2).

All proteins containing LRRs identified to date are thought to be involved in protein-protein interactions². Although different LRR proteins bind a variety of unrelated ligands, the basic principles of ligand binding learned from the RNase A-RI complex can be transferred to other proteins with LRRs.



The two unusual features of the structure of RI are its non-globular shape and the solvent-exposed parallel β -sheet. The structure of the RNase A-RI complex shows that these two features are successfully used in this particular interaction. The non-globular shape allows an extensive binding area for a smaller protein ligand such as RNase A and thus the formation of many favourable contacts. The solvent-exposed parallel β -sheet is a rare feature in protein structures¹⁶⁻¹⁸; our results show that it can be successfully used as a protein-binding motif. In RI, its surface is built almost entirely of well packed side chain atoms (in the free RI⁴, only two residues from the central 15 strands have over 0.2 Å² of the backbone exposed to a 1.4 Å probe; the average B -factor of the side-chain atoms is 20.0 Å² in the β -sheet and 34.4 Å² for the rest of the molecule). This makes the use of side chains in the interaction entropically less unfavourable and allows changes in specificity by modifying the surface through amino-acid substitutions.

TABLE 2 Contacts between RNase A and RI in the complex

Residue	RNase A:	K7	Q11*	N24	Q28	K31	S32	L35	D38	R39*	K41*	P42	V43*	K66*	N67*	Q69*	N71*	E86	G88	S89	S90	K91	A109	E111*	H119*
RI																									
H6						V																			
C7							V																		
D31						V																			
N89			H, V	V																					
N117			H, V																						
E202																			H, V						
D228																			V						
W257																			H				V		
W259																			V						
E283																				H, V				V	
W314																						V		V	
K316																			H						
E397									H, V																
C404													H, V		V										
V405														H, V											
G406														V											
D407																									
Q426										V															
V428										V															
Y430									H	V															
D431											V														
Y433												V													
W434																									
T435																									
E436																									
R453																									
I455									H, V																
S456									V																
			H, V	H																					

Putative hydrogen bonds with donor-acceptor distances below 3.5 Å are indicated with 'H' and van der Waals contacts with distances below 4 Å are indicated with 'V'. One-letter amino-acid code is used.

* RNA-binding residues of RNase A⁷⁻⁹ that contact RI in the RNase A-RI complex.

The conformational flexibility of RI suggests that LRRs do not simply provide a stable platform for the display of specific determinants for a recognition process; instead, they provide a flexible platform that can adjust to requirements of a particular interaction. Another example of such conformational flexibility has been observed in antibodies where the variable domains can slightly change their relative orientation upon antigen binding⁹. □

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Tubular membrane invaginations coated by dynamin rings are induced by GTP-γS in nerve terminals

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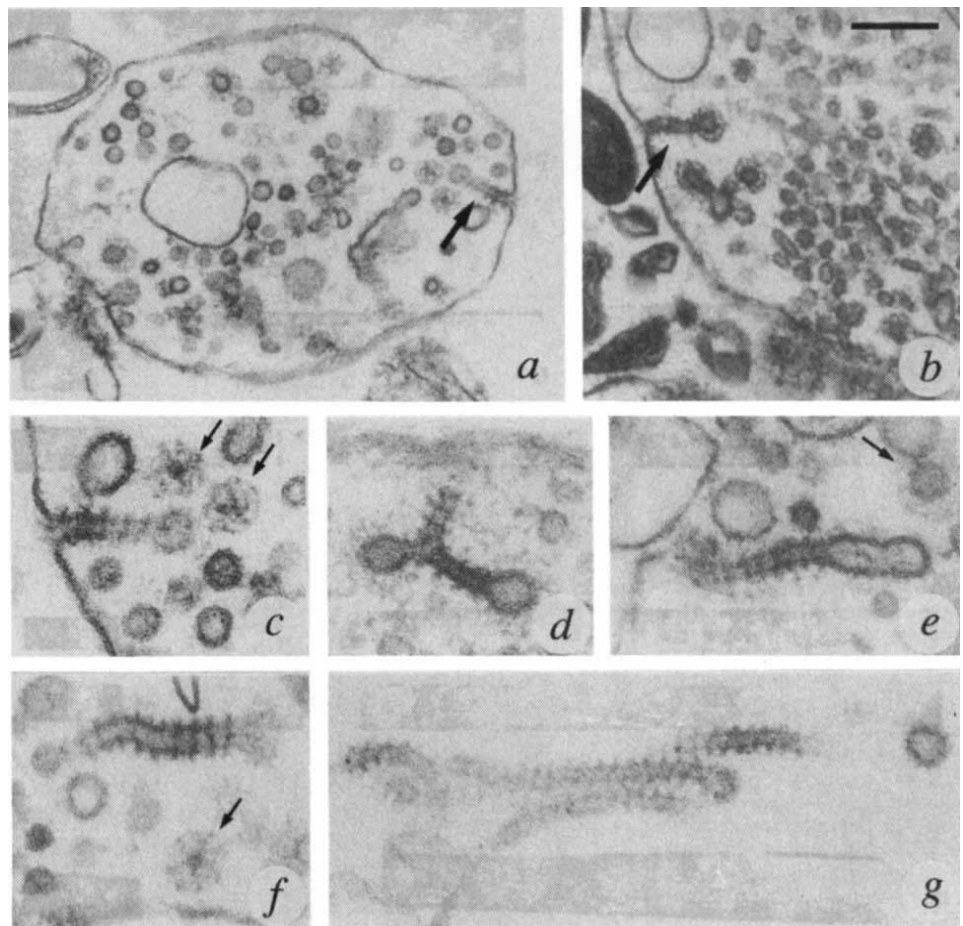
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THE mechanisms through which synaptic vesicle membranes are reinternalized after exocytosis remain a matter of debate¹⁻⁵. Because several vesicular transport steps require GTP hydrolysis⁶⁻⁹, GTP-γS may help identify intermediates in synaptic vesicle recycling. In GTP-γS-treated nerve terminals, we observed tubular invaginations of the plasmalemma that were often, but not always, capped by a clathrin-coated bud. Strikingly, the walls of these tubules were decorated by transverse electron-dense rings that were morphologically similar to structures formed by dynamin around tubular templates^{10,11}. Dynamin is a GTPase implicated in synaptic vesicle endocytosis¹²⁻¹⁴ and here we show that the walls of these membranous tubules, but not their distal ends, were positive for dynamin immunoreactivity. These findings demonstrate that dynamin and clathrin act at different sites in the formation of endocytic vesicles. They strongly support a role for dynamin in the fission reaction and suggest that stabilization of the GTP-bound conformation of dynamin leads to tubule formation by progressive elongation of the vesicle stalk.

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FIG. 1 Electron micrographs demonstrating the presence of tubular membrane invaginations coated by regularly spaced striations in nerve terminal membranes incubated with GTP- γ S. *a-f*, Hypotonically lysed nerve terminals from a rat brain synaptosomal fraction (lysed P_2). *a* and *b*, Low-power views of two nerve terminals. Coated tubular invaginations of the plasmalemma, which terminate in a clathrin-coated bud, are indicated by thick arrows. *c-f*, Examples of tubular invaginations at higher magnification (field *c* is a high magnification of the tubule visible in *a*). The blind end of the tubule shown in *e* is uncoated. Clathrin-coated vesicles are indicated by thin arrows. *g*, A cluster of coated tubules present in a synaptosomal membrane subfraction (LP_2). Scale bar, *a* and *b*, 220 nm; *c-g*, 100 nm. METHODS. Membrane fractions (P_2 and LP_2) were prepared from rat brain homogenates as described²⁷. The P_2 fraction, which is enriched in intact nerve terminal membranes (synaptosomes), was lysed by the addition of 9 vol of ddH₂O. LP_1 and LP_2 were generated from the lysed P_2 fraction by sequential low-speed and high-speed centrifugation steps. The use of LP_1 and LP_2 membrane subfractions allowed for better access of exogenously added cytosolic components and nucleotides to nerve terminal membranes. Lysed P_2 and LP_2 fractions were resuspended in 'cytosolic buffer'⁶ (25 mM HEPES-KOH pH 7.4, 25 mM KCl, 2.5 mM magnesium acetate, 5 mM EGTA, 150 mM K-glutamate). Membrane fractions (100 μ l) were added to 730 μ l of 'cytosol' (see below) and then taken to 1 ml by the addition of the compounds described below to obtain the following final concentrations: 2 mM ATP, 16.7 mM creatine phosphate, 16.7 IU ml⁻¹ creatine phosphokinase, 200 μ M GTP- γ S. These mixtures were incubated for 15 min at 37 °C. Controls were done by replacing GTP- γ S with GTP or by incubating the membrane fractions with cytosolic buffer alone, cytosolic buffer plus cytosol and cytosolic buffer plus cytosol and ATP.



A gallery of electron micrographs taken from lysed nerve terminals that had been incubated in the presence of cytosol, ATP and GTP- γ S are shown in Fig. 1*a-f*. Several examples of membranous tubules decorated with regularly spaced rings are visible. The rings sometimes appeared obliquely arranged, suggesting a helix. In addition, clathrin-coated vesicles and invaginated clathrin-coated pits were abundant in these preparations (Fig. 1*a-f*, and data not shown). In favourable sections, tubules, which were about 25–30 nm in diameter, were generally seen to connect uncoated clathrin-coated vesicles to the plasmalemma (Fig. 1*a, b, c*). In a few cases, however, the end of the tubules did not appear to be clathrin coated (Fig. 1*e*). The formation of these coated tubular structures was dependent on GTP- γ S because similar structures were not observed in samples reacted with cytosol alone, cytosol + ATP, or cytosol + ATP and GTP (not shown). Tubules were also observed in GTP- γ S-incubated membrane subfractions derived from lysed nerve terminals (Fig. 1*g*). In this case, tubules were very frequently observed, tended to be longer and occasionally formed small bundles or clusters (Fig. 1*g*).

The striations seen around the membranous tubules were very similar in morphology to structures formed *in vitro* by purified dynamin around microtubules^{10,11}. This similarity suggested that dynamin may form the striations, an attractive

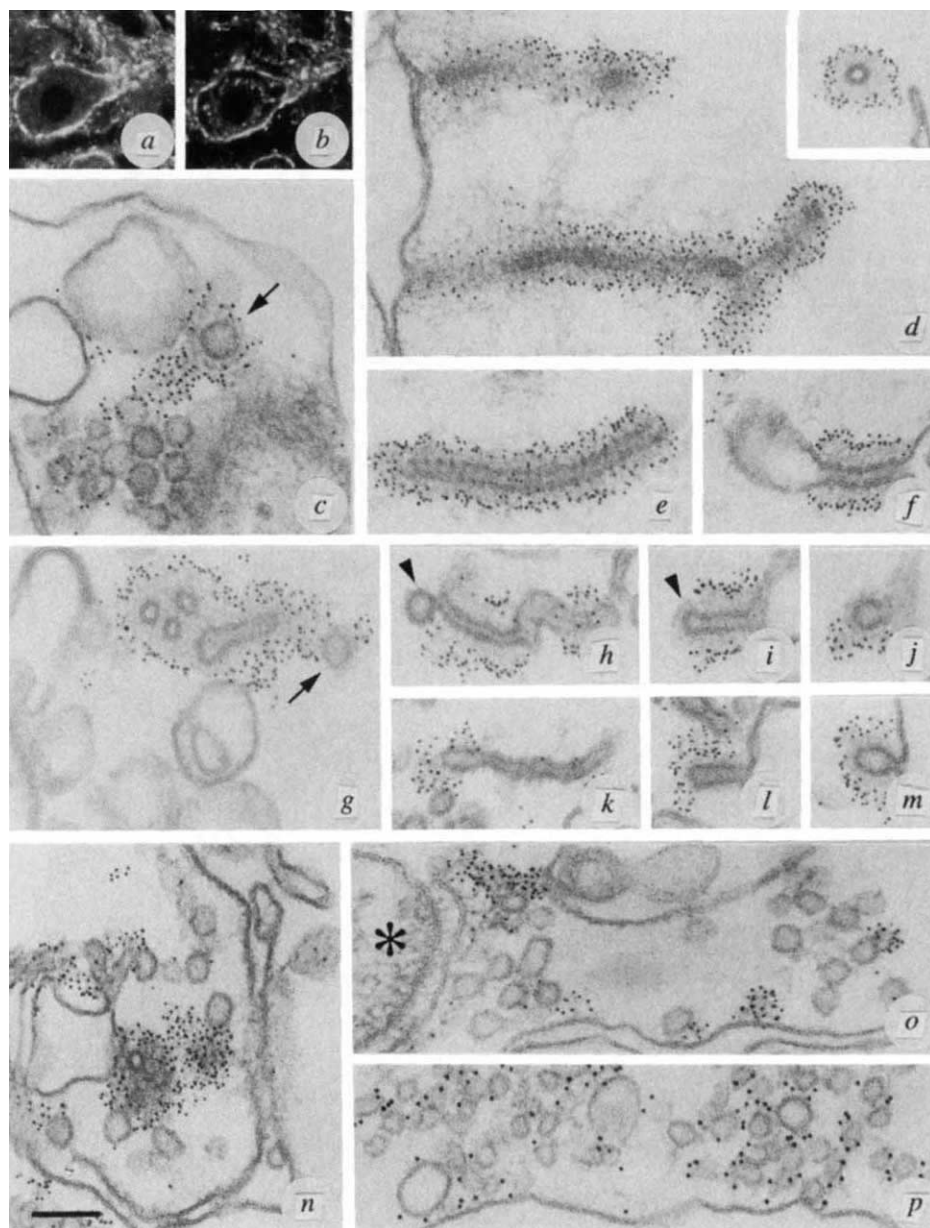
The incubations were stopped by adding 1 ml of 2 $\times$ concentrated fixative (final concentrations: 3% formaldehyde, 2% glutaraldehyde, 0.32 M sucrose in HEPES-KOH buffer). To prepare cytosol, an S_3 fraction prepared as described^{27,28} was desalted with Sephadex G-25M PD-10 column (Pharmacia Biotech). Fixed samples were analysed by electron microscopy using standard procedures. Images from fields *a-f* were taken from preparations impregnated with 1% tannic acid⁶ to enhance visualization of the coat.

possibility given the putative role of dynamin in endocytosis. In *Drosophila*, temperature-sensitive mutations of the dynamin gene (*shibire*) lead to a temperature-sensitive impairment in endocytosis of synaptic vesicle membranes^{13–17}. In fibroblasts, expression of GTP-binding domain mutants of dynamin blocks receptor-mediated endocytosis^{18,19}. Invaginated clathrin-coated pits accumulate in HeLa cells expressing mutant dynamin and both wild-type and mutant dynamin have been shown by immunogold electron microscopy to be localized to clathrin-coated pits²⁰.

GTP- γ S-treated membranes were therefore labelled for dynamin by immunogold electron microscopy using a monoclonal anti-dynamin antibody previously shown to produce an intense staining of nerve endings by immunofluorescence²¹ (Fig. 2*a, b*). As shown in Fig. 2*d-i*, an extremely intense and specific gold labelling of the cytoplasmic surface of the tubular structures was observed. Dynamin labelling was less concentrated and sometimes absent at the distal end of the tubules (Fig. 2*f, h, i*). In contrast, immunogold labelling for clathrin resulted in a heavy labelling of the distal end of most tubules, but not of their shaft (Fig. 2*k, l*). In addition, clathrin immunolabelling produced a heavy decoration of coated pits (Fig. 2*m*) and vesicles (not shown). Patches of dynamin immunolabelling were generally present near these organelles (Fig. 2*c, g, j*) (see also ref. 20), but

FIG. 2 Coated membrane tubules are intensely immunoreactive for dynamin and negative for clathrin. **a**, Light microscopy immunofluorescence pattern of the anti-dynamin antibodies used for EM studies. Immunoreactivity is concentrated in nerve terminals which outline the surface of perikarya. **b**, Counterstain of the same section with anti-clathrin antibodies which produce a similar staining pattern in nerve terminals but which also label the Golgi complex (perinuclear fluorescence)²⁹. **c–j**, Electron micrographs demonstrating immunogold labelling for dynamin of nerve terminal membranes incubated with GTP- γ S. **c** is from a lysed crude synaptosomal fraction (lysed P_2). **d–f** are from low-speed (LP_1) and **e–j** are from high-speed (LP_2) pellets of the lysed P_2 fraction. Anti-dynamin immunogold is highly concentrated around the shaft of the tubules which are completely surrounded by gold particles (see cross-sections in the inset of **d** and in **g**), but is much less concentrated or is absent at the tips of the tubules (arrowheads in **h** and **i**). Patches of anti-dynamin labelling are visible adjacent to clathrin-coated vesicles (**c** and **g**, arrows) and pits (**j**), but anti-dynamin immunogold does not homogeneously decorate the clathrin coat. **k–m**, Anti-clathrin immunolabelling of an LP_2 fraction incubated with GTP- γ S. Anti-clathrin immunogold is absent from the tubules (**k** and **l**) but homogeneously decorates the blind end of the tubules and coated pits (**m**). **n** and **o**, Hypotonically lysed nerve terminals immunolabelled for dynamin immediately after lysis. Sparse patches of immunogold are present among the unlabelled synaptic vesicles. One such patch is localized around a cross-sectioned tubule (**n**). Asterisks in **o** indicate the post-synaptic density. **p**, Lysed nerve terminal labelled for synaptophysin as a control. In these preparations immunogold is localized evenly in the nerve terminal around synaptic vesicles. Scale bar, **a** and **b**, 28 μ m; **c–p**, 100 nm.

METHODS. Immunofluorescence was as described³⁰. For electron microscopy immunolabelling, lysed P_2 , LP_1 and LP_2 fractions (see methods in Fig. 1) (**c–m**) were incubated in GTP- γ S-containing media and fixed as described in the legend of Fig. 1 with the exception that the final aldehyde concentrations in the fixative were 3% paraformaldehyde and 0.2% glutaraldehyde. Fields **n–p** were obtained from rat brain homogenates prepared in hypotonic conditions and immediately fixed as described³⁰. Fixed fractions were then processed for agarose



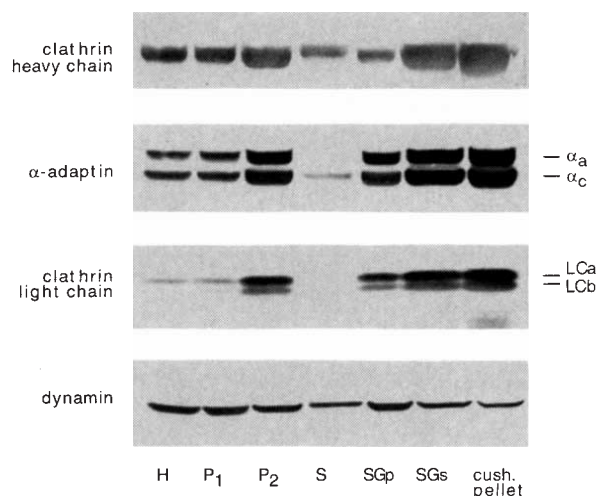
embedding, immunogold labelling and electron microscopy essentially as described^{30,31}. Dynamin and clathrin immunolabelling was done by sequential incubations with mouse monoclonal antibodies, rabbit anti-mouse antibodies and protein A-gold. Synaptophysin immunolabelling was as described³². Monoclonal anti-dynamin antibodies^{20,21} and polyclonal antibodies directed against a neuron-specific insert of clathrin light chain²⁹ (**b**) were previously described. Monoclonal antibodies against clathrin light chain (Cl 57.1) (**k–m**) were a kind gift of R. Jahn.

failed to decorate their surface homogeneously as observed for anti-clathrin immunolabelling.

In control nerve terminals, fixed after lysis without earlier incubation with cytosol and nucleotides, the bulk of dynamin immunoreactivity was observed at clathrin-coated pits and in clusters adjacent to clathrin-coated vesicles²⁰ as well as in patches over the cytomatrix which may represent the reserve pool of dynamin not involved in endocytosis (Fig. 2*n, o*). However, a few examples of gold particle clusters, which appeared to surround small membrane tubules, have been observed (Fig. 2*n*). These tubules were not identified in preparations not immunolabelled for dynamin, probably because of their very low num-

ber. The presence of tubular invaginations ending in clathrin-coated buds were previously described in nerve terminals during the recovery period after intense stimulation¹. Furthermore, images of clathrin-coated vesicles connected to the plasmalemma by long and narrow tubules (25 nm in diameter) similar to those described here, were also observed in non-neural cells²². These observations raise the possibility that GTP- γ S may shift the equilibrium of a reaction that occurs in the living cell, although the precise relationship between membrane tubules induced by GTP- γ S and tubules observed in intact cells remains to be elucidated.

To validate further the conclusion that dynamin and clathrin



act at distinct sites in coated vesicle formation, we compared the distribution of clathrin and dynamin during subcellular fractionation of rat brain in a procedure leading to purified clathrin-coated vesicles. Although dynamin was present in purified clathrin-coated vesicles in agreement with immunogold observations (ref. 20 and this study), there was less dynamin per unit protein in the coated vesicle fraction than in total homogenate (Fig. 3). In contrast, clathrin light and heavy chains, as well as α -adaptins²³, were highly enriched in purified coated vesicles (Fig. 3).

Our study demonstrates that GTP- γ S induces tubular endocytic invaginations in nerve terminals. The membrane tubules are decorated by evenly spaced, often slightly oblique, electron-dense rings which suggest the thread of a screw, and dynamin is a major component of these rings. The similar structures that purified dynamin forms around isolated microtubules^{10,11} are also stabilized by GTP- γ S¹¹, supporting the hypothesis that coated tubular structures reported here result from a direct effect of GTP- γ S on dynamin. However, we cannot rule out the possibility that GTP- γ S may have a role in the formation of the

FIG. 3 Association of dynamin with clathrin-coated vesicles. Aliquots from various steps of a subcellular fractionation procedure leading to purified rat brain clathrin-coated vesicles were immunoblotted with antibodies directed against proteins involved in endocytosis as indicated on the left. The dashes on the right indicate the migratory position of α -adaptin isoforms (α_a and α_c) or clathrin light chain isoforms (LCa and LCb). H, Homogenate; P, pellet; S, supernatant; SGp, sucrose gradient pellet; SGs, sucrose gradient supernatant; cush. pellet, purified clathrin-coated vesicles. Anti-adaptin antibodies were a kind gift from M. Robinson.

METHODS. Clathrin-coated vesicles from rat brain were prepared as described²⁹. Equal amounts of protein from each subcellular fraction (100 μ g) were separated on SDS-PAGE, transferred to nitrocellulose, and western blots were done as previously described³³. Antibodies against α -adaptin³⁴ have been characterized elsewhere. Other antibodies were described in the legend of Fig. 2.

screw-like structures by acting through a GTP-binding protein other than dynamin. Although the physiological significance of the binding of dynamin to microtubules and its property to form regular arrays around microtubules *in vitro* remains unknown^{11,24}, our present work describes similar arrays at sites where dynamin is thought to act. Our observations are therefore likely to be closely related to the function of dynamin. The diameter of the membrane tubules is similar to the diameter of microtubules (25 nm) and it is possible that the structures formed by dynamin around microtubules may highlight its property to polymerize around templates which contain dynamin-binding sites. Physiologically, such a template may be represented by the neck of a coated pit. In view of our findings, it is quite likely that dynamin is a component of the collar of dense material that can be seen around the neck of invaginated pits in nerve terminals of *shibire* flies at the restrictive temperature^{15,16}. We note that the diameter of these necks¹⁶ is similar (20–25 nm) to that of the GTP- γ S-induced tubules.

These results, which are complemented by the demonstration that purified dynamin can form rings *in vitro*³⁵, strongly support a model in which the invagination of clathrin-coated vesicles and their fission from the plasmalemma are driven by different molecular mechanisms⁸, and strongly indicate that dynamin participates selectively in the fission process, which is known to require GTP hydrolysis²⁵ (Fig. 4). They also provide clues concerning the mechanisms by which the connection between constricted coated vesicles and the donor membrane is severed. The dynamin ring (or helix) may provide a scaffold which, upon a conformational change (for example, constriction, twist) dependent upon GTP hydrolysis could force membrane fusion at the neck of the vesicle. Stabilization of the GTP-bound conformation of dynamin by GTP- γ S leads to progressive polymerization of dynamin and elongation of the vesicle neck to form a tubule. Downregulation of dynamin's GTPase activity in the intact cell, for example by dephosphorylation^{21,26}, may play a role in the formation of the tubular endocytic invaginations observed *in situ*¹. Finally, the presence of tubules not capped by a clathrin coat raises the possibility that dynamin, possibly with other associated proteins, may be sufficient to generate a tubular invagination and therefore to support endocytosis in a clathrin-independent fashion. □

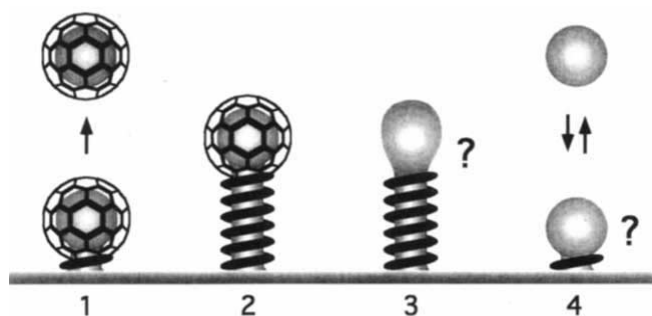


FIG. 4 Model of possible interpretations of the dynamin localizations demonstrated in this paper. Dynamin oligomers are proposed to form a collar at the neck of invaginated clathrin-coated pits. A conformational change of dynamin which correlates with GTP hydrolysis leads to vesicle fission (1). GTP- γ S, by stabilizing the GTP-bound conformation of dynamin, leads to tubule formation by progressive elongation of the vesicle stalk (2). *In situ*, an increase in the GTP-bound conformation of dynamin may be mediated by the downregulation of dynamin's GTPase activity, perhaps by dephosphorylation^{21,26}. The presence of dynamin-coated tubules not capped by a clathrin-coated end (this study) (3) and of invaginated pits not coated by clathrin in nerve terminals of *shibire* flies at the restrictive temperature¹⁶ (4) may be due to clathrin loss or to endocytic events which are clathrin-independent (for example as postulated in the 'kiss and run' model of neurotransmitter release³).

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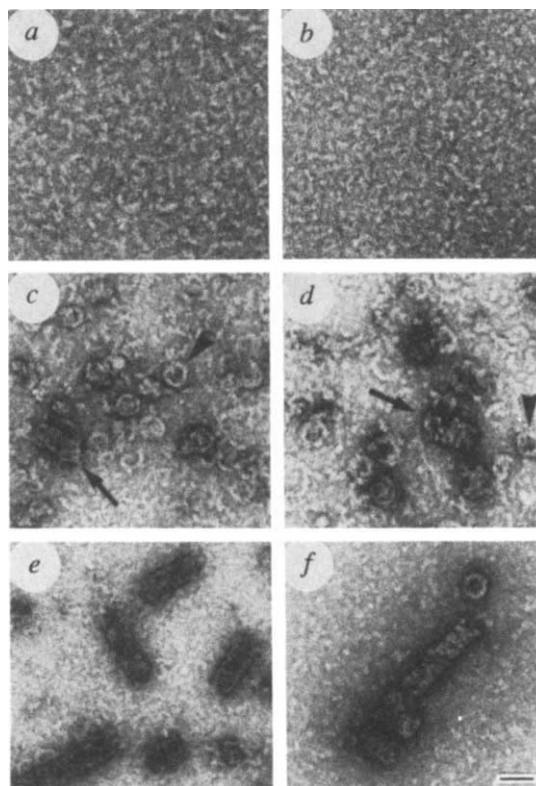


FIG. 1 Low-magnification negative-stained images of assembled and unassembled dynamin oligomers. *a*, Intact dynamin oligomeric assembly units or *b*, subtilisin-digested dynamin in HCB300 (see methods). *c*, Wild-type dynamin or *d*, K44A mutant dynamin in HCB300 were diluted sixfold into HCB lacking NaCl before analysis by negative-stain electron microscopy. The protein concentration after dilution was $\sim 0.4 \text{ mg ml}^{-1}$. Partial rings, rings (arrow-heads) and small stacks of rings (arrows) are seen in both samples. *e*, Stacks of rings which are prevalent after dialysis of intact dynamin ($>1 \text{ mg ml}^{-1}$) into HCB containing 25 mM NaCl. *f*, Subtilisin-treated dynamin stacks. All images are shown at the same magnification. Scale bar, 100 nm.

METHODS. Dynamin was purified from Sf9 insect cells infected with recombinant baculovirus encoding wild-type dynamin or the K44A mutant dynamin as described¹⁴. In the final purification step, dynamin was eluted from a Superose 6 FPLC column (Pharmacia) in HCB300, HEPES column buffer (20 mM HEPES, pH 7.2, 2 mM MgCl₂, 1 mM EDTA, 14 mM DTT) containing 300 mM NaCl, well resolved from lower *M_r* contaminants. Limited proteolysis was done at a ratio of 1 μg subtilisin (Sigma) to 400 μg dynamin for 20 min on ice and the digestion stopped by addition of 1 mM phenylmethylsulphonyl-fluoride (PMSF). This incubation produced a stable $\sim 90\text{K}$ digestion product of dynamin in high yield. Samples were diluted to $\sim 0.1 \text{ mg ml}^{-1}$, adsorbed to carbon-coated EM grids and negatively stained with 2% uranyl acetate before air drying. For examination, a Philips CM12T electron microscope was used at 100 kV with a 70 μm objective aperture.

experiments (not shown) indicated that dynamin exists as an elongated molecule probably consisting of four 100K polypeptides. To elucidate its structure further, purified dynamin was examined by negative-stain electron microscopy. Elongated structures, $\sim 10 \text{ nm}$ wide and $\sim 20\text{--}40 \text{ nm}$ long with varying degrees of curvature, presumably corresponding to dynamin tetramers, were observed (Fig. 1*a*).

The C-terminal proline-rich domain (PRD) of dynamin is the site of interaction with a variety of activators of dynamin's GTPase activity, including microtubules and grb2⁵. To determine if this region is also involved in dynamin–dynamin interactions within the oligomers, limited proteolysis by subtilisin was used to produce a $\sim 90\text{K}$ polypeptide lacking the proline-rich domain^{8,16}. Subtilisin-treated dynamin also eluted from a

Dynamin self-assembles into rings suggesting a mechanism for coated vesicle budding

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DYNAMIN, a 100K member of the GTPase superfamily¹, is the mammalian homologue of the *Drosophila shibire* gene product^{2,3}. Mutations in *shibire* cause a defect in endocytosis leading to accumulation of coated pits and deep invaginations at the plasma membrane of all tissues examined^{4,5}. Similarly, invaginated coated pits accumulate in mammalian cells overexpressing dominant-negative mutants of dynamin, establishing that dynamin is required for the formation of 'constricted' coated pits and for coated vesicle budding⁶. Whether dynamin functions in the classic GTPase mode as a molecular switch to regulate events leading to coated vesicle budding or instead actively participates as a mechanochemical enzyme driving coated vesicle formation is unclear⁷. Here we show that dynamin spontaneously self-assembles into rings and stacks of interconnected rings, comparable in dimension to the 'collars' observed at the necks of invaginated coated pits that accumulate at synaptic terminals in *shibire* flies⁴. We propose that invaginated coated pits become constricted by the assembly of dynamin into rings around their necks. A concerted conformational change would then close the rings and pinch off the budding coated vesicles.

Neuronal dynamin was purified to homogeneity from Sf9 insect cells infected with recombinant baculovirus encoding human dynamin-1¹⁶. The $\sim 100\text{K}$ dynamin polypeptide eluted after gel-filtration chromatography on Superose-6 with an apparent *M_r* (assuming a globular protein) in excess of 900K (not shown). Velocity sedimentation analysis and crosslinking

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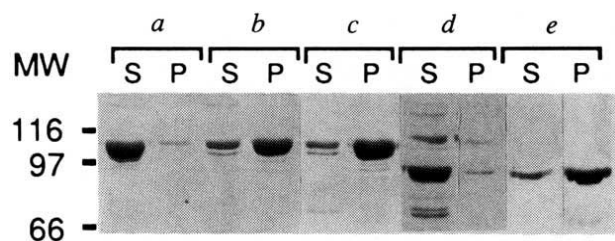
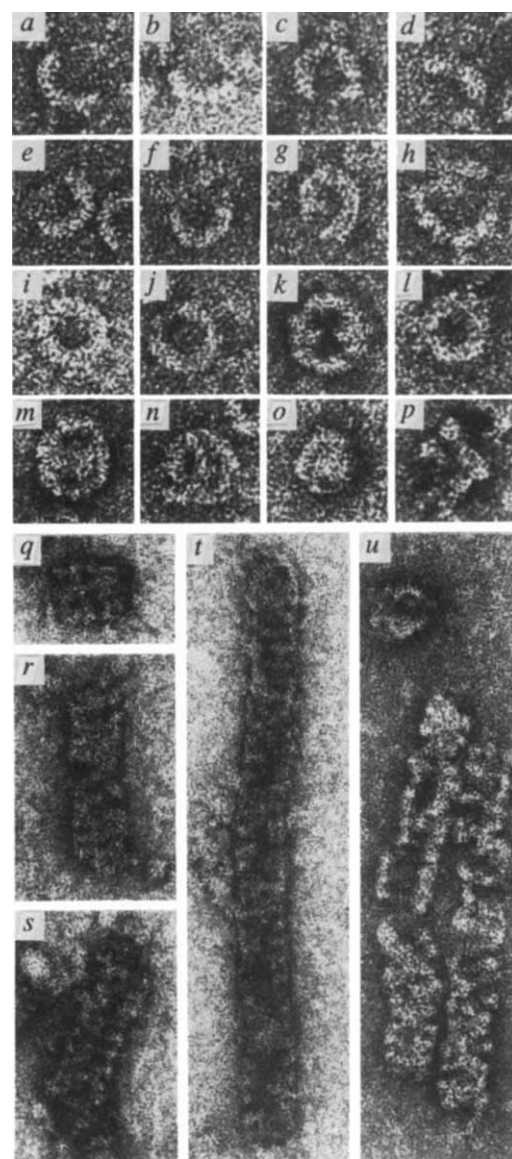


FIG. 2 A simple sedimentation assay for dynamin self-assembly. SDS-PAGE analysis of the supernatants (S) and pellets (P) obtained by centrifugation at 50,000 r.p.m. for 15 min in a TLA100 rotor (Beckman Instruments) of dynamin subjected to various incubation conditions. Intact wild-type dynamin (a and b), K44A mutant dynamin (c), or subtilisin-digested wild-type dynamin (d) at $\sim 2.5 \text{ mg ml}^{-1}$ in HCB300 were diluted sixfold into either HCB300 (a) or HCB0 (b–d) 15 min before centrifugation. e, Intact dynamin ($\geq 1.0 \text{ mg ml}^{-1}$) was dialysed against HCB25 to form sedimentable rings and stacks which were then incubated in the same buffer with subtilisin for 20 min on ice as described in Fig. 1 before addition of PMSF and centrifugation.

FIG. 3 A gallery of structures formed upon spontaneous assembly of dynamin. a–d, Individual oligomeric assembly units of dynamin. The image in c suggests the end-to-end assembly of individual oligomers to form partial rings. e–h, Partial rings formed by end-to-end assembly of individual oligomers. The curvature of these partial rings varied significantly. h, A single assembly unit just above a partial ring consisting apparently of 2–3 assembly units. i–l, Examples of 'hollow' or 'open' rings. Overlapping of partial rings, with material extending into the centre, can be seen in k. m–p, 'Filled' or 'closed' rings. n, A spiral with tight curvature suggesting a mechanism for ring closure. p and q, Edge-on views with two or three rings forming a small stack. r–u, Long stacks of rings. r, The helical nature of the stacks can be seen suggesting that adjacent rings are interconnected. At high-protein concentrations very long stacks can be formed (t). u, A subtilisin-digested stack where partial unravelling is apparent. These images suggest interconnections between adjacent rings with a helical twist. Scale bar, 50 nm.



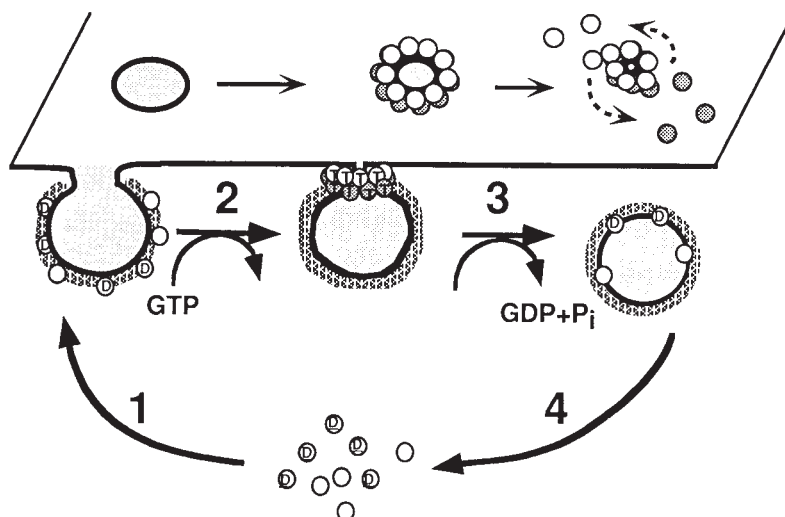
Superose-6 column as an apparent oligomer with molecular mass of $\sim 500\text{K}$. Hydrodynamic and crosslinking characteristics of subtilisin-cleaved dynamin were most consistent with its being a dimer (not shown). As expected, the structures observed in negative-stained images of subtilisin-treated dynamin were smaller than those observed for intact dynamin (compare Fig. 1b and a). These data suggested that dynamin's PRD is required for native oligomerization.

When dynamin molecules, isolated in 300 mM NaCl-containing buffers, were diluted or dialysed into lower salt (25–50 mM NaCl) they spontaneously self-assembled into a mixture of partial-rings, intact rings and small stacks of interconnected rings (Fig. 1c). These structures were largely sedimentable, providing a quantitative assay for dynamin self-assembly. In high-salt buffers only $\sim 13\%$ of dynamin sedimented (Fig. 2a), however, upon dilution into low salt, up to $\sim 83\%$ of dynamin formed sedimentable structures (Fig. 2b). Dialysis of dynamin in low salt (maintaining a higher protein concentration) resulted in the formation of striking tubules of dynamin formed from stacks of rings (Fig. 1e). Similar helical arrays of dynamin have been observed bound to microtubules^{9,10}, but here we show that purified dynamin is capable of this self-assembly reaction in the absence of a polymeric substrate. Assembly was reversible: when returned to 300 mM salt, the dynamin stacks disassembled and reverted to the elongated and curved structures seen in Fig. 1a (not shown).

Guanine nucleotide binding was not required for dynamin self-assembly. When a dynamin mutant that cannot bind or hydrolyse GTP^{6,11} (designated K44A), was diluted or dialysed into low-salt conditions, its self-assembly into sedimentable rings and stacks was indistinguishable from wild-type dynamin (Figs 1d and 2c). In contrast, dynamin self-assembly was completely prevented following subtilisin-digestion to remove its PRD (Fig. 2d). Once assembled, removal of the C-terminal PRD by subtilisin did not destabilize the sedimentable rings and stacks (Figs 1f and 2e). Therefore, the PRD is required for self-assembly but not for maintaining assembled rings or stacks. Other regions of dynamin that might participate in stabilizing these structures include the 'self-assembly' domain (amino acids 46–80, referring to the human dynamin sequence) defined for the dynamin-related Mx proteins¹², the coiled-coil domain (amino acids 718–768) and the pleckstrin homology domain (amino acids 510–633)¹³, all of which have been implicated as participating in protein–protein interactions. The quantitative removal of the PRD from the sedimentable rings and stacks by subtilisin digestion established that this region is accessible for interactions with other proteins even when dynamin is assembled.

A gallery of high-magnification images of assembled dynamin, which suggests a mechanism for dynamin assembly into rings and then stacks, is shown in Fig. 3. We define the elongated structures prevalent in high-salt buffers as dynamin 'assembly

FIG. 4 A working model for the role of dynamin self-assembly in coated vesicle formation. Circles represent dynamin in its unoccupied (open), GDP-bound (indicated by 'D'), or GTP-bound (indicated by 'T') forms. Our model proposes that dynamin is recruited to coated pits in its unoccupied or GDP-bound forms in step 1. In step 2, GTP binding induces the redistribution of dynamin and its self-assembly into a 'collar' which constricts the necks of invaginated coated pits. 'Constricted' coated pits remain connected to the plasma membrane and can be functionally distinguished from 'invaginated' coated pits as ligands sequestered within them are inaccessible to macro-molecular probes but accessible to small molecules¹⁵. A cross-section, drawn roughly to scale, is shown above. In step 3, GTP hydrolysis causes a conformational change, closing the ring structure (see cross-section) to drive coated vesicle budding. Coincident disassembly of the rings releases dynamin for recycling (step 4). See text for details.



units' (Fig. 3a-d). The partial rings seen in Fig. 3(e-h) were most prevalent at lower concentrations of dynamin ($<0.5 \text{ mg ml}^{-1}$) or at intermediate salt concentrations ($\sim 100 \text{ mM}$). Considerable flexibility in the oligomeric dynamin structure is suggested by the observation of both slightly curved (Fig. 3h) and tightly curved (Fig. 3g) structures. Complete rings were observed with either open (Fig. 3i-l) or apparently closed lumens (Fig. 3m-p), again suggesting flexibility. From edge-on or tilted images it appeared that ring closure might be helped by the formation of a tight spiral or 'lock-washer' structure (Fig. 3n, p and q). The open rings had an outer diameter of $\sim 50 (\pm 10) \text{ nm}$ and an inner diameter of $\sim 30 \text{ nm}$. The width of the rim, 10 nm , was the same as for individual assembly units suggesting that the rings were assembled from 4-6 dynamin assembly units in an end-to-end fashion although the C-terminal PRD remains accessible. Finally, individual rings can apparently assemble into well ordered stacks which were most prevalent and longer at higher concentrations of dynamin ($>1 \text{ mg ml}^{-1}$) (Fig. 3q-t). The stacks had variable numbers of rings ranging from 2 or 3 (Fig. 3p, q) to greater than 30 (Fig. 3t). Upon closer observation, particularly of the subtilisin-treated dynamin which have partially unravelled (Fig. 3u), the rings were seen to be interconnected with a pronounced helical twist.

A model for the role of dynamin self-assembly in clathrin-coated vesicle budding is shown in Fig. 4. The model derives from the structural analysis of dynamin presented here, our previous studies on the guanine nucleotide requirements for late stages in coated vesicle formation *in vitro*¹⁴, and our functional analysis of wild-type and mutant dynamin molecules *in vivo*⁶. These combined data suggest that dynamin is recruited in either its GDP-bound or unoccupied states from the cytosol to coated pits on the plasma membrane (Fig. 4, step 1), where initially it

is diffusely distributed throughout the clathrin lattice⁶. Because the formation of 'constricted' coated pits *in vitro* requires GTP but not GTP hydrolysis¹⁴, we propose (Fig. 4, step 2) that GTP binding and/or GTP-GDP exchange (perhaps controlled by dynamin accessory proteins) triggers the redistribution of dynamin from the clathrin lattice to the necks of invaginated coated pits where it self-assembles into collars. The dimensions of the open rings and of the dynamin-encircled tubules which accumulate in GTP- γ S-treated nerve terminals¹⁷ are consistent with the idea that this self-assembly would result in the formation of a 'constricted' coated pit. Because GTP- γ S inhibits coated vesicle budding *in vitro*¹⁴ (Fig. 4, step 3), GTP hydrolysis could result in a concerted conformational change in dynamin that tightens the rings or stacked structures around the constricted necks driving membrane fission and vesicle budding. Further structural studies will be required to elucidate the nature of the conformational change that closes the ring structures.

In conclusion, we have established that dynamin joins clathrin as the second structural protein to undergo a self-assembly reaction in mediating coated vesicle formation. Because dynamin self-assembly *in vitro* appears not to require guanine nucleotides, we propose that GTP binding *in vivo* regulates dynamin's interactions with as yet unidentified 'partners' that regulate and target its assembly, under physiological salt conditions, into collars around the necks of invaginated coated pits. Other partners, perhaps through interactions with the accessible PRD, might then regulate GTP hydrolysis and the concerted conformational changes required to tighten the rings and sever the necks. By analogy to the proteins that regulate clathrin assembly and disassembly, the factors that regulate dynamin self-assembly and disassembly will be central to elucidating the mechanism of its function. □

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A kinase–cyclin pair in the RNA polymerase II holoenzyme

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THE RNA polymerase II holoenzyme consists of RNA polymerase II, a subset of general transcription factors, and regulatory proteins known as SRB proteins^{1,2}. The genes encoding SRB proteins were isolated as suppressors of mutations in the RNA polymerase II carboxy-terminal domain (CTD)^{3,4}. The CTD and SRB proteins have been implicated in the response to transcriptional regulators^{1–11}. We report here the isolation of two new *SRB* genes, *SRB10* and *SRB11*, which encode kinase- and cyclin-like proteins,

respectively. Genetic and biochemical evidence indicates that the *SRB10* and *SRB11* proteins form a kinase–cyclin pair in the holoenzyme. The *SRB10/11* kinase is essential for a normal transcriptional response to galactose induction *in vivo*. Holoenzymes lacking *SRB10/11* kinase function are strikingly deficient in CTD phosphorylation. Although defects in the kinase substantially affect transcription *in vivo*, purified holoenzymes lacking *SRB10/11* kinase function do not show defects in defined *in vitro* transcription systems, suggesting that the factors necessary to elicit the regulatory role of the *SRB10/11* kinase are missing in these systems. These results indicate that the *SRB10/11* kinase is involved in CTD phosphorylation and suggest that this modification has a role in the response to transcriptional regulators *in vivo*.

To identify new components of the RNA polymerase II holoenzyme, we isolated extragenic suppressors of a *Saccharomyces cerevisiae* RNA polymerase II CTD truncation mutation⁴. Recessive suppressing mutations were identified in two genes, *SRB10* and *SRB11*. Genetic analysis indicated that the CTD and the two *SRB* gene products are involved in the same process in transcription initiation. The mutant alleles *srb10-1* and *srb11-1* suppressed the conditional phenotypes of CTD truncation mutations but not the conditional phenotypes of other RNA polymerase II mutations. Genomic DNA clones containing *SRB10* and *SRB11* were isolated by genetic complementation and the complementing clones with the smallest inserts were sequenced (Fig. 1).

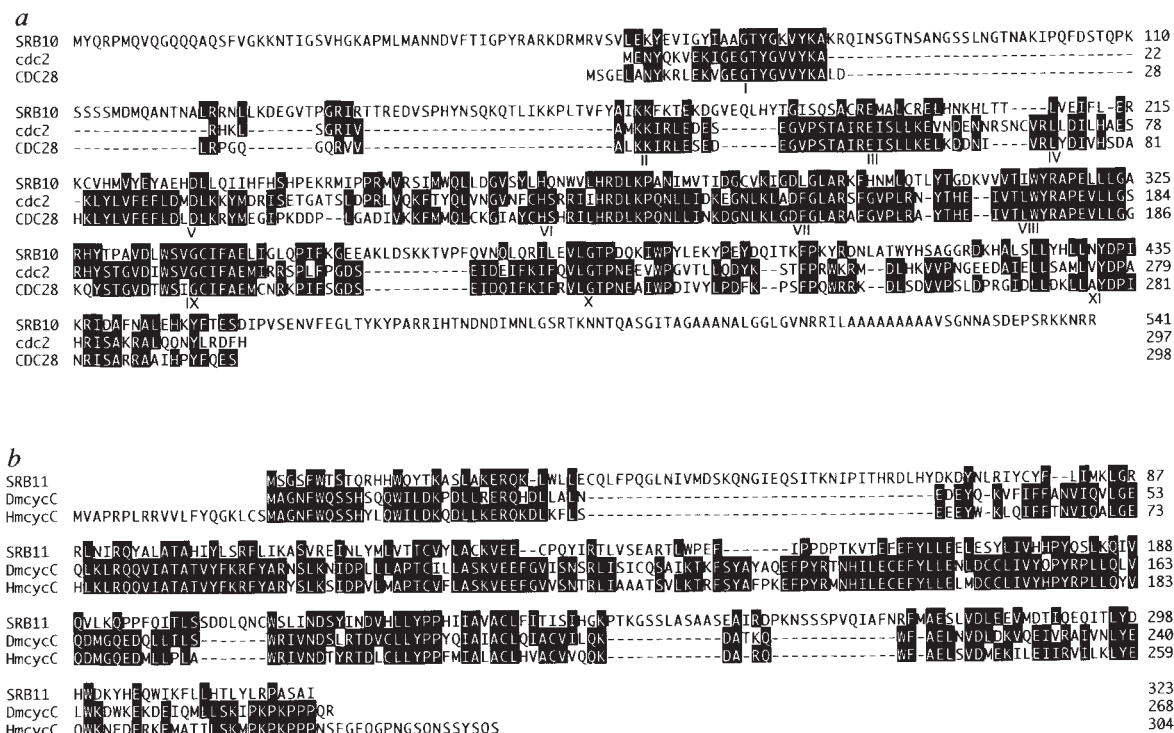


FIG. 1 Sequence of *SRB10* and *SRB11* proteins and alignment with related kinases and cyclins. a, *SRB10* sequence alignment with the *S. pombe* Cdc2 and *S. cerevisiae* CDC28 proteins. Identical amino-acid residues are shown as white letters on a black background. Roman numerals indicate the 11 conserved kinase subdomains. Primer extension analysis of poly(A)⁺ mRNA revealed that the *SRB10* transcript is initiated at position +12 in the open reading frame originally predicted for *UME5* (ref. 13). Thus the amino-acid sequence of *SRB10/UME5* shown here lacks the 14 amino-terminal amino acids originally reported for *UME5*¹³. b, *SRB11* sequence alignment with *Drosophila* (*DmcyoC*) and human (*HmcyoC*) cyclin C proteins.

METHODS. Genomic DNA clones containing *SRB10* and *SRB11* were isolated by exploiting their ability to reverse the suppressing phenotype of the recessive *srb* alleles in cells containing the truncated CTD allele (*rpb1Δ104*). A wild-type genomic DNA library constructed in a yeast

URA3 centromeric plasmid⁴ was transformed into yeast cells containing the CTD truncation mutation *rpb1Δ104* and *srb10-1* or *srb11-1*. *Ura*⁺ transformants were then screened for phenotypes characteristic of the *rpb1Δ104* CTD truncation mutant: lack of growth at 12 °C and inability to use pyruvate as a carbon source. Genomic clones containing *SRB10* (pSL201) and *SRB11* (pJ211-1) were sequenced. *SRB10* and *SRB11* were physically mapped to chromosome XVI (λ clones 3168 and 4122) and to chromosome XIV (λ clone 3634), respectively, using the prime λ clone grid filters of the yeast genome (from L. Riles and M. Olson). Sequence comparison and alignments were made with the BLAST network service and the DNA Star Megalign program, respectively. The nucleotide sequence data for *SRB10* and *SRB11* can be found in the EMBL, GenBank and DDBJ nucleotide sequence databases under the accession numbers U20222 and U20221, respectively.

SRB10 encodes a 541-amino-acid protein kinase related to the Cdc2 kinases¹² (Fig. 1a). *SRB10* is identical to *UME5*, described recently as a regulator of meiosis-specific genes¹³. *SRB10/UME5* protein sequence is ~40% identical to the *S. cerevisiae* CDC28 and *S. pombe* Cdc2 proteins and highly similar to PHO85, CTK1 and KIN28 (refs 14–16, respectively), members of the yeast Cdc2-related subfamily of serine/threonine protein kinases.

SRB11 encodes a 323-amino-acid protein which is ~28% identical and 58% similar in sequence to the human¹⁷ and *Drosophila*¹⁸ cyclin C proteins (Fig. 1b). Residues 75–184 of *SRB11* are 37% identical to those of the 'cyclin box' of the human and *Drosophila* proteins; this region is conserved in cyclins and may be involved in the interaction between kinase and cyclin subunits^{19–21}.

We investigated whether *SRB10* and *SRB11* could be components of the RNA polymerase II holoenzyme. Western blot analysis with anti-*SRB10* antibodies revealed that essentially all of the *SRB10* in cell extracts copurified with the RNA polymerase II holoenzyme at each step of the purification procedure. The results in Fig. 2a show that RNA polymerase II and the *SRB2*,

SRB4, *SRB5*, *SRB6* and *SRB10* proteins co-elute in the final purification step of the holoenzyme. Because we were unable to generate anti-*SRB11* antibodies, an independent purification was carried out using a lysate from cells containing epitope-tagged *SRB11*; all of the epitope-tagged *SRB11* copurified precisely with the RNA polymerase II holoenzyme (Fig. 2a). Thus, essentially all of the *SRB10* and *SRB11* in cell extracts is associated with the holoenzyme. Immunoprecipitation experiments confirmed that *SRB10* and *SRB11* are tightly associated with the holoenzyme (Fig. 2b). Thus, genetic and biochemical analysis indicates that all six *SRB* proteins identified so far (*SRB2*, 4, 5, 6, 10 and 11) are components of the transcription initiation complex, the RNA polymerase II holoenzyme.

The sequences of *SRB10* and *SRB11*, together with genetic evidence that the two gene products are involved in the same function, indicate that they may form a kinase-cyclin pair. The recombinant proteins bound to one another on a column (Fig. 2c) and interacted in a two-hybrid system²² (data not shown), indicating that *SRB10* and *SRB11* are components of a kinase-cyclin pair in the holoenzyme.

FIG. 2 *SRB10* and *SRB11* are components of the RNA polymerase II holoenzyme. **a**, RNA polymerase II holoenzyme was purified as described¹. Holoenzyme loaded onto a Mono-S column, the last chromatographic step in the purification procedure, was eluted with a 0.1–1.0 M gradient of potassium acetate. The output (OP) and flow through (FT) and a portion of every other fraction eluting between 0.1 and 0.9 M potassium acetate were analysed for holoenzyme activity (top panel) and for the presence of RNA polymerase II and *SRB* proteins by western blot analysis. The western blot for *SRB11* was done with an RNA polymerase II holoenzyme purified independently from cells with an epitope-tagged *SRB11* protein; the purification and transcriptional properties of this holoenzyme were identical to the holoenzyme lacking the epitope tag. **b**, Co-immunoprecipitation of *SRB4*, *SRB10* and *SRB11* with *SRB5*. Purified RNA polymerase II holoenzyme was immunoprecipitated using affinity-purified anti-*SRB5* or anti-HSP70 antibodies. The supernatant (S), wash (W) and precipitate (P) were analysed by western blotting using antibodies against specific *SRB* proteins. Lanes: 1 and 4, supernatant from immunoprecipitation with anti-*SRB5* and anti-HSP70 antibodies; 2 and 5, washes of immunoprecipitated material; 3 and 6, precipitated material. **c**, Recombinant *SRB10* interacts with a glutathione-S-transferase (GST)-*SRB11* fusion protein in affinity chromatography. Radiolabelled *SRB10* or CDC28 was produced in an *in vitro* transcription/translation system and loaded onto GST-*SRB11* and GST columns. Fractions were subjected to SDS-PAGE. OP, 0.5% of output; FT, 0.5% of flowthrough; W, 10% of final wash; E, 10% of elution with reduced glutathione.

METHODS. Holoenzyme purification and *in vitro* transcription assays have been described¹. The mAb 8WG16 (Promega) was used to detect RPB1 in western blots, rabbit anti-*SRB* antibodies to detect *SRB2*, *SRB4*, *SRB5*, *SRB6* and *SRB10*, and mAb 12CA5 to detect an influenza haemagglutinin (HA) epitope tag introduced at the N terminus of *SRB11*²⁶. Bands were visualized by secondary probing with alkaline-phosphatase-conjugated secondary antibodies (Pierce) or by chemiluminescence with horseradish-peroxidase-conjugated secondary antibody (Amersham). Immunoprecipitations were done with holoenzyme purified from an HA-tagged *SRB11* strain (Z689) in buffer containing 50 mM HEPES-KOH, pH 7.3, 15 mM magnesium acetate, 100 mM potassium acetate, 1 mM EGTA, 10% glycerol, 0.1 mM DTT, 0.1% NP-40. Anti-rabbit antibody linked to dynabeads (Dyna) was used as the secondary reagent in the immunoprecipitation. For column chromatography, a GST-*SRB11* fusion was constructed (pMV123) using pGEX-2T (Pharmacia) and recombinant GST-*SRB11* and GST were purified from *E. coli* as described²⁷. Columns containing 250 μ l GST-beads were loaded with 40–50 μ g GST-*SRB11* or GST, and equilibrated with transcription buffer⁸. 25 μ l labelled *in vitro* translated *SRB10* or CDC28 (Promega TNT coupled system) was incubated with the immobilized proteins overnight at 4 °C, washed with transcription buffer +1% Triton X-100, and eluted with 10 mM reduced glutathione, 50 mM Tris-HCl, pH 8, 1% TritonX-100, 0.1 mM PMSF. Figures were prepared from digital replicas of primary data scanned using a UMAX UC840 Max Vision digital scanner.

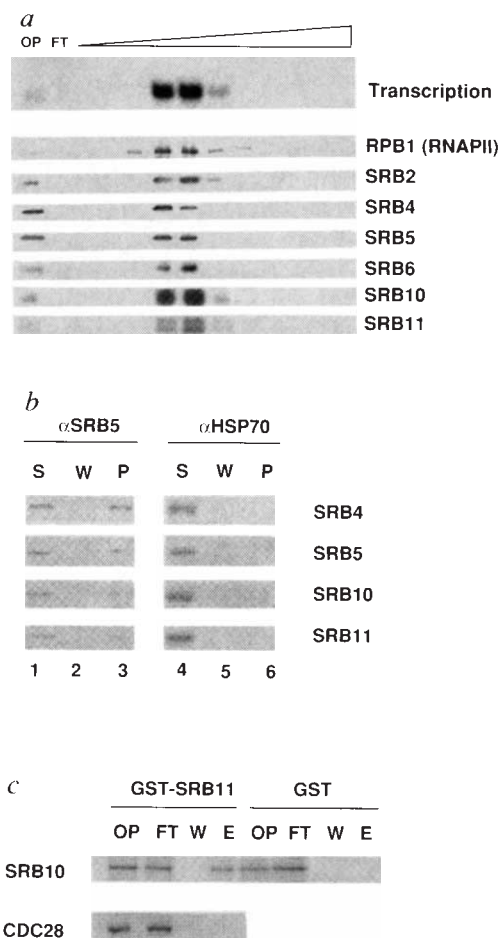
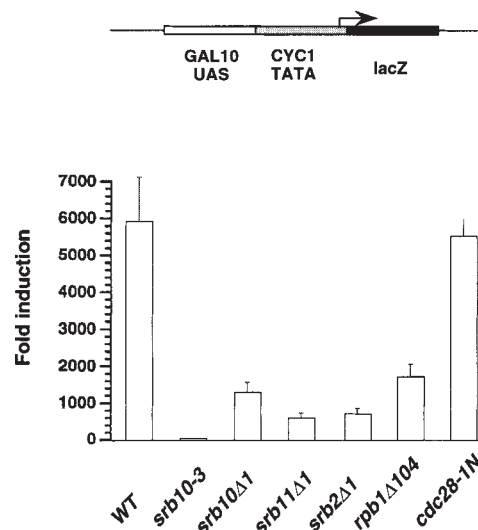


FIG. 3 *SRB10* and *SRB11* mutants are deficient in transcriptional responses at a *GAL10* promoter *in vivo*. The relative ability of wild-type cells and *srb10* and *srb11* mutants to respond to galactose induction was measured by expressing *lacZ* under the control of a *GAL10* UAS-containing promoter, as described⁶. Extracts were prepared from induced or uninduced cells and the results of β -galactosidase assays are expressed as fold induction. Strains assayed were wild type (WT); Z690 (*srb10-3*) cells encoding an *SRB10* protein in which Asp 290 had been replaced with Ala; Z687 (*srb10 Δ 1*) cells in which *SRB10* is deleted; Z688 (*srb11 Δ 1*) cells in which *SRB11* is deleted; Z437 (*srb2 Δ 1*) cells in which *SRB2* is deleted; Z551 (*rpb1 Δ 104*) cells encoding an RNA polymerase II CTD truncation mutant with 11 heptapeptide repeats; L5191 (*cdc28-1N*) cells encoding a mutant form of *CDC28*. METHODS. The *GAL10* UAS–*CYC1*–*lacZ* fusion plasmid pLGDS5, galactose induction procedures and β -galactosidase assay have been described⁶. Complete deletions of the *SRB10* and *SRB11* coding sequences (*srb10 Δ 1* and *srb11 Δ 1*) were constructed using a single-step disruption method²⁸. The gene encoding the *SRB10* D290A mutation (*srb10-3*) was constructed by oligonucleotide mutagenesis, and the Z690 mutant was generated by replacing the wild-type *SRB10* gene with *srb10-3*, as described²⁸. The *srb10* and *srb11* mutant strains are conditionally viable; they exhibit mild cold-sensitive, temperature-sensitive, and slow growth phenotypes.



Mutations in the RNA polymerase II CTD and in *SRB2* reduce the response of the transcription apparatus to regulators *in vivo*^{3–7}, so we investigated the effects of mutations in *SRB10* or *SRB11* *in vivo* (Fig. 3). A β -galactosidase reporter vector with a *GAL10* UAS-containing promoter was introduced into cells with CTD and *srb* mutations and the cells were subjected to induction by galactose. The results revealed that cells lacking *SRB10* or *SRB11* function respond poorly to galactose. The defect was most pronounced (100-fold) in cells containing a point mutation (*srb10-3*) that inactivates the kinase function

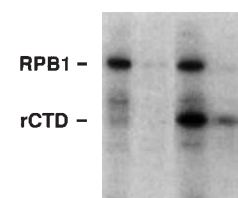
of *SRB10* without affecting its stable incorporation into the holoenzyme. We infer that the defect is in transcriptional induction rather than in messenger RNA stability because mutations in *SRB10/UME5* do not destabilize vegetative mRNAs and actually increase meiotic mRNA stability twofold¹³. *SRB10* and *SRB11* mutant cells are slow growing, but control experiments show that slow growth does not inhibit induction by galactose (Fig. 3). The poor response of *srb10* and *srb11* mutant cells to transcriptional induction *in vivo* is consistent with the association of these two gene

FIG. 4 CTD phosphorylation and transcription *in vitro* with wild-type and mutant RNA polymerase II holoenzymes. *a*, Purified holoenzyme lacking *SRB10* function exhibits reduced CTD phosphorylation. Wild-type (W) and mutant (M) holoenzymes were assayed for their ability to phosphorylate the CTD of the RNA polymerase II large subunit or recombinant CTD added to the holoenzyme preparations. The mutant RNA polymerase II holoenzyme contains *SRB10* protein in which Asp 290 has been replaced with Ala. Phosphorylation of the endogenous RNA polymerase II CTD was reduced ~10-fold and ~5-fold for recombinant CTD, in the mutant holoenzyme. *b*, Western blots showing that the two holoenzyme preparations contain similar amounts of the largest subunit of RNA polymerase II (RPB1) and *SRB* proteins. *c*, Wild-type and *SRB10* mutant holoenzymes exhibit similar basal and activated transcription *in vitro*. Lanes: 1, wild-type holoenzyme (W); 2, wild-type holoenzyme + GAL4–VP16; 3, *SRB10* mutant holoenzyme (M); 4, *SRB10* mutant holoenzyme + GAL4–VP16. The large transcript is derived from the pGAL Δ template, which lacks a GAL4 binding site, and the small transcript is from pGAL $\times$ 6, which contains six GAL4-binding sites.

METHODS. RNA polymerase II holoenzymes were purified from wild-type and *srb10-3* mutant cells as described¹. Kinase assays were carried out at 24 °C with 100 ng holoenzyme in 15 μ l buffer containing 20 mM HEPES, pH 7.6, 8 mM MgSO₄, 2.5 mM EGTA, 5% glycerol, 2 mM DTT and a mixture of phosphatase inhibitors (1 mM NaN₃, 1 mM NaF, 0.4 mM NaVO₃, 0.4 mM NaVO₄ and 0.1 mg ml⁻¹ phosphitin). Recombinant GST–CTD (100 ng) was added to a subset of the reactions; GST itself is not phosphorylated by holoenzyme preparations (not shown). Transcription was performed as described²⁹ with the following modifications: 100 ng pGAL Δ and pGAL $\times$ 6 templates³⁰ were used per reaction; factors and DNA were preincubated for 15 min before adding nucleotides; and the stop mixture contained 12.5 U ml⁻¹ ribonuclease T1. TFIIE was prepared as described²⁹ except that the second BioRex column was omitted and a Mono-S column step was added at the end.

a Kinase assay

Holoenzyme:	W	M	W	M
rCTD:	-	-	+	+

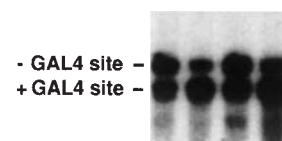


b Western blots

Holoenzyme:	W	M
RPB1	—	—
SRB2	—	—
SRB5	—	—
SRB10	—	—

c Transcription

Holoenzyme:	W	W	M	M
GAL4-VP16:	-	+	-	+



products with CTD function, which was previously implicated in such responses.

The RNA polymerase II CTD is found in an unphosphorylated form in transcription initiation complexes but is extensively phosphorylated during elongation^{11,23}. Thus, CTD phosphorylation may regulate some event in transcription initiation. We tested whether SRB10/11 kinase has a role in CTD phosphorylation in the holoenzyme using purified RNA polymerase II holoenzyme from wild-type and *srb10* mutant cells. Figure 4 shows that CTD phosphorylation was reduced ~10-fold in the *srb10* mutant holoenzyme, indicating that the SRB10 kinase must be important for CTD phosphorylation; the reduced response of SRB10-deficient holoenzyme to galactose induction *in vivo* may therefore reflect its diminished ability to phosphorylate the CTD. The yeast general transcription factor TFIIF is present in the holoenzyme¹, and the TFIIF-associated kinase^{24,25} may account for the residual CTD phosphorylation in the mutant holoenzyme.

The activities of wild-type and SRB10-mutant RNA polymerase II holoenzymes were compared in a reconstituted transcription assay (Fig. 4c); levels of basal and GAL4-VP16-activated transcription were similar for both holoenzymes. We could not find any defect in transcription *in vitro* comparable to the loss of CTD phosphorylation *in vitro* or of galactose induction *in vivo*. These results suggest that factors necessary to elicit the regulatory role of SRB10 are missing or not functional in our *in vitro* transcription systems. Alternatively, the holoenzymes may contain additional kinases that compensate for the loss of SRB10 function in these systems.

We have identified a kinase-cyclin pair in the RNA polymerase II holoenzyme, shown that it is involved in transcriptional regulation *in vivo* and in CTD phosphorylation *in vitro*. Although the exact role of CTD phosphorylation in transcriptional regulation is not known, our results demonstrate that the response of the transcription initiation apparatus to at least some regulatory signals *in vivo* involves the SRB kinase-cyclin pair. □

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ERRATA

Dimercaptan–polyaniline composite electrodes for lithium batteries with high energy density

N. Oyama, T. Tatsuma, T. Sato & T. Sotomura

Nature **373**, 598–600 (1995)

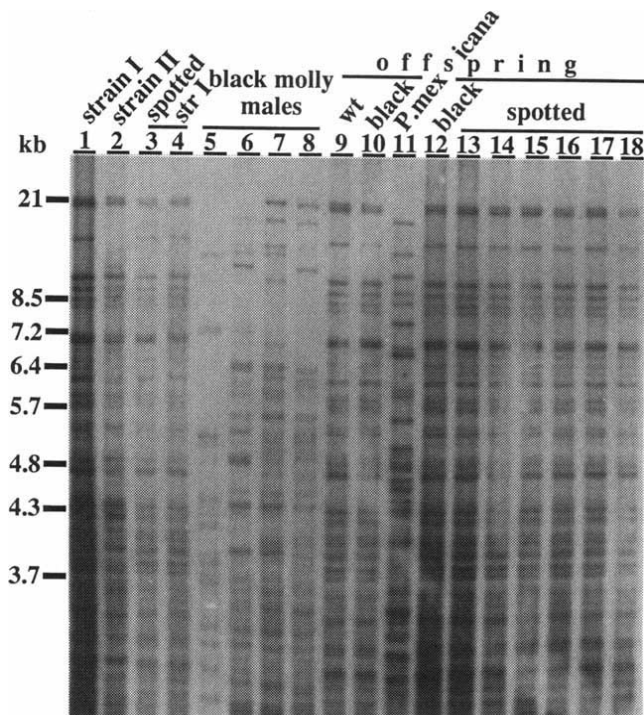
FIGURES 3 and 4 were accidentally transposed in this Letter. □

Incorporation of subgenomic amounts of DNA as compensation for mutational load in a gynogenetic fish

Manfred Schartl, Indrajit Nanda, Ingo Schlupp, Brigitta Wilde, Jörg T. Epplen, Michael Schmid & Jakob Parzefall

Nature **373**, 68–71 (1995)

IN Fig. 2A of this Letter, the designation of lanes above the gel was incomplete. The correct labelling is shown here. For example, lane 11 contains DNA from *Poecilia mexicana*, which was used as host species to produce the offspring, and not DNA from an offspring, as would have been inferred from the incomplete labelling. □



Making waves with liquid handling

For your liquid handling consideration — a series of robotic sample processors, a rheological characterization instrument, a multichannel pump system and solvent-safe pipettes.

KONTES has developed a new **solvent recovery system** for the protection of employee health and the environment (*Reader Service No. 100*). These systems are designed to eliminate up to 98 per cent of the toxic organic solvent vapour emissions from the extraction laboratory, to assist with emission law and waste-reduction requirements, and in the minimization of background contamination from extraction solvents associated with gas chromatography and gas chromatography/mass spectroscopy analyses. Packages include extraction glassware, heating equipment and vapour recovery components — turnkey systems are also offered. Systems are also adaptable to existing water baths, steam tables and heating mantles in any configuration required.

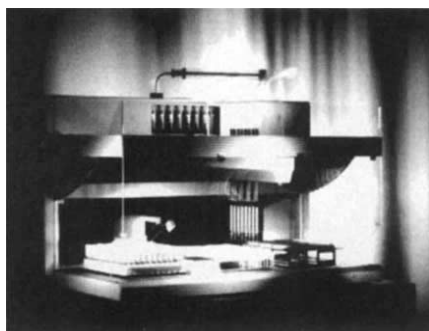
Haake now offers the RT10 to accommodate all test methods for the **rheological characterization of fluids** (*Reader Service No. 101*). The RT10 can perform the controlled rate method for hysteresis curves on thixotropic substances, with speed control from 0.1 to 1,000 min^{-1} and ramp programmes. For creep tests and measurements at small shear rates down to 10^{-6} s^{-1} the instrument offers controlled stress with torque control up to 8 Ncm. The oscillation method quantifies the viscoelastic behaviour of materials over a range from 0.01 to 10 Hz. A variety of measuring geometries are available: the concentric cylinder sensor (Searle) with geometry according to DIN53018, DIN 54543, DIN 53019 and ISO 3219; cone and plate sensors according to ISO 3219; and parallel plates with adjustable gap and profiled sensors for slippage. Also available are double cone and double plate geometries for low-viscosity fluids, a pressure measuring sensor for up to 3,500 kPa and 200 °C, disposable sensors (cup and rotor) made out of aluminium and optically transparent sensors for rheo-optic tests. The RT10 provides liquid temperature control over a number of temperature ranges.

■ Automated workstations

Zymark has designed the Autoplate-96 workstation to improve throughput and to eliminate the well-to-well variability of reagent delivery in **microtitre plate pipetting** (*Reader Service No. 102*). The pipettor aspirates and dispenses in microlitre increments and can pipette volumes from 1 μl to 200 μl from reagent reservoirs, deep wells and microplates. Disposable pipette tips prevent cross-contamination and carryover. The workstation's turntable handles six

microplates or deep-well plates. Windows software allows adjustment and optimization of pipetting variables for procedures, including plate replications, reagent additions and serial dilutions.

The new Genesis series of **robotic sample processors** (RSPs) has been developed by Tecan to meet current and future requirements in automated liquid handling (*Reader Service No. 103*). This is a modular series of instruments with four or eight pipetting probes available, providing throughputs of up to 1,500 samples each hour. Any combination of Teflon-coated stainless steel tips and disposable tips (200 μl and 1,000 μl with or without filters) can be selected. Both series of disposable tips can be used on the same run without changing the system hardware. The Genesis series comes with both horizontal and vertical barcodes to identify and locate all



Tecan's Genesis series RSPs.

items on the worksurface, including primary and secondary tubes and plates. The sensitivity of liquid-level detection is adjustable for optimized performance both with conductive and a range of non-conductive liquids — including DMSO. The force of vertical tip movement is controlled to allow piercing of caps on tubes but prevent injury to operators. A Windows environment controls the system and software packages dedicated to particular applications are available. The worksurface is designed for flexibility: microplates, deep-well plates, tube racks, sample tube strips, reagent bottles, disposable tip racks and a range of other carriers can be used.

The new **autosampler system** based on the Gilson XL has been introduced by ATI Unicam for use with its ultraviolet/visible series of spectrometers (*Reader Service No. 104*). The system is designed for high throughput and can be used with the ultraviolet series local keypad or with PC-based Windows software. The system enables results to be generated in simple absorbance, against a calibration curve, or

in scanning applications. Communication between the personal computer and the instrument allows results to be processed while the instrument is in operation.

Omega's new PHP300 series programmable, solenoid-driven diaphragm **liquid metering pumps** can be operated manually or automatically, utilizing a 4 mA–20 mA input or pulse input (*Reader Service No. 105*). The stroke length of the pump is adjustable from 10 to 100 per cent, while the stroking rate is adjustable from 12 to 125 strokes per minute. Head and filling material options include glass-filled polypropylene, polyvinylidene fluoride, and AISI-316 stainless steel. The diaphragm is made of Teflon-faced Hypalon. Guided check valves are designed to provide precise seating and priming and suction lift characteristics.

■ Pumps and syringes

Watson-Marlow has introduced the 205 series of **multichannel pumps** designed for near pulseless microlitre flows through 4 to 32 separate channels at four-channel increments (*Reader Service No. 106*). The 205S and 205U pumps are fitted with robotics-quality direct current servo motors and include microprocessor control to increase accuracy and ensure smooth, quiet operation. Flow rates range from 0.2 $\mu\text{l min}^{-1}$ to 32 ml min^{-1} . Additional features include rapid priming, a low-speed setting to keep lines open and an automatic restart feature that allows pumps to recover automatically from an electrical failure. Large, backlit display panels and lockable keypads offer easy and secure operation. A choice of three pumpheads allows the user to upgrade their requirements at any time. Cassettes can be added or removed from pumpheads without disturbing the flow of any other channel, and accept a full range of colour-coded manifold tubing, available in Marprene, PVC, silicone and solvent- or acid-resistant materials.

ProMinent Fluid Controls has developed a self-bleeding liquid end for the gamma/4 series of **precision metering pumps** (*Reader Service No. 107*). This facility is intended for applications where the pump may be switched off for long periods of time, overnight for example. It is also useful where the pumping fluids either decompose to produce gases or trap gas bubbles within them. This new liquid end features a degassing bypass diaphragm valve with a spring-loaded ball. Any air or gas from the suction line is automatically removed via the bypass valve, ensuring that

the dosing chamber is filled with fluid to achieve accurate metering. The liquid end is interchangeable with standard dosing heads, and the gas bypass valve removes the need to have a separate back pressure valve when dosing at low outlet or low back-pressures.

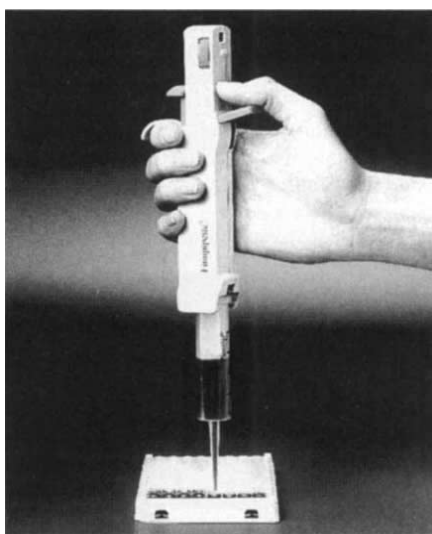
To overcome the problems caused in the pumping of corrosive gases and vapours, Vacuubrand has developed the RC5 hybrid pump (*Reader Service No. 108*). The design combines a rotary vane and a diaphragm pump in one unit. The oil-sealed part of a modified rotary vane pump is evacuated by a corrosion-resistant diaphragm pump. This is intended to prevent corrosion or condensation within the oil sealed part of the pump. The RC5 operates without a cooling trap and reduces oil wastage. The pump has a flow-rate of $5.1 \text{ m}^3 \text{ h}^{-1}$.

The PSD/2, from Hamilton, is an integrated, self-contained precision syringe drive module that can be configured and used as a single unit or linked with multiple units in a daisy chain (*Reader Service No. 109*). The PSD/2 utilizes surface-

mount components, and a single mould (rigid chassis platform) for compactness. The inert HV valves can be changed with a snap-in/snap-out movement. The fluid path of the PSD/2 is inert, coming into contact with PTFE, FEP, CTFE and glass. A daisy-chain of up to 16 modules can be assembled with automatic addressing for each module. Two communication protocols exist for this unit.

■ Pipetting

LabSystems has introduced the Finnpiptette Stepper for repeat pipetting for up to 45 times in succession without refilling (*Reader Service No. 110*). A range of seven tip sizes is available to cover a volume range from $10 \mu\text{l}$ to $5,000 \mu\text{l}$. The Stepper is designed to be



LabSystems Finnpiptette Stepper.

lightweight and ergonomic for user comfort. The company states that one-handed operation is easy with the finger rest and dispensing lever. This is a positive displacement pipette and is said to be suitable for 'aggressive' and viscous liquids.

Four new Pastette transfer pipettes are available from Alpha Laboratories (*Reader Service No. 111*). The cell-saver Pastette has been specifically designed for cell biology techniques. The large tip aperture is designed to allow safe pipetting of cell cultures without damaging the cells. It is also useful for pipetting viscous liquids. The large bulb micro Pastette has a capacity of 15 ml while the narrow stem allows access to various containers. It can also be heat sealed and used as a transport, storage or reaction vessel. Also new is the fine-tip Pastette with an extended fine tip and $20 \mu\text{l}$ drop size — intended for small-volume transfers and delicate dispensing work. The final member of the family is the long-reach Pastette, shaped to remove liquid from spectrophotometric cuvettes without scratching.

The activated carbon filter found in Molecular Bio-Products's solvent-safe pipette tips is now available in a 1-ml to 5-ml 'macro' pipette tip size (*Reader Service No. 113*). These tips are designed to stop corrosive fumes generated while pipetting acids, bases and volatile organic compounds — useful for phenol/chloroform extractions, HPLC experiments and peptide synthesis. Solvent-safe tips are intended to reduce the frequency of pipettor servicing and protect pipette barrels from becoming brittle and breaking. These tips allow the use of an air-displacement pipettor as an alternative to positive placement and serological pipetting systems.

■ Odds and ends

The Nalgene PassPort is a packaging system suitable for the transport of hazardous material (*Reader Service No. 114*). These pretested, combination packaging systems are designed for single use and feature Nalgene IP2 plastic bottles as the inner containers. PassPort is designed to assist with US Department of Transportation HM-181 compliance. PassPort shipping systems are said to meet International Civil Aviation Organization, International Air Transportation Association and International Maritime Organization regulations for Package Group I. Hazardous materials from 125 ml up to 4 l can be shipped using one of the 32 single-bottle and four-pack configurations; with narrow- and wide-mouth bottles.

Upchurch Scientific now offers colour-coded, translucent Teflon tubing to assist with system organization and maintenance (*Reader Service No. 115*). This material also allows the visualization of mobile phase flow. Black Teflon tubing is offered for use in light-sensitive applications. The tubing is designed to be inert and of high quality. The 1.6 mm outer-diameter tube is pressure rated to 6,891 kPa and the 3.2 mm outer-diameter tubing is rated to 3,445 kPa.

The Varispenser and Varispenser plus, from Eppendorf are designed for safe and economical bottle-top dispensing (*Reader Service No. 116*). The Varispenser plus safety-valve system recirculates the reagent in a closed circuit to remove air. This is intended to provide bubble-free dispensing. A multichannel valve at the end of the discharge tube is designed to prevent dripping and wastage of expensive reagents. Both models are autoclavable and are designed for reproducible dispensing of 0.5 ml to 50 ml. □

These notes are compiled by Brendan Horton from information provided by the manufacturers. For more details, fill in the reader service card bound inside the journal.

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